

REMARKS/ARGUMENTS

Upon entry of this amendment, claims 1, 4, 7, 10 and 13 are pending in the application. Claims 2-3, 5-6, 8, 9, 11-12 and 14-18 have been canceled without prejudice to subsequent revival. Claim 1 has been amended. Entry of the amendment, reconsideration of the rejection, and allowance of claims 1, 4, 7, 10 and 13 are respectfully requested.

The Amendment

In order to expedite prosecution of the application and advance the case toward allowance, the claims have been amended. No new matter was added by the amendment.

Claim 1 has been amended to specify that the condition is selected from the group consisting of rheumatoid arthritis, alveolitis and atherosclerosis. Support for this amendment can be found in the specification, for example, in paragraph 0006. Therein, the Applicants disclose that MCP-1 has been implicated as an important factor in mediating monocytic infiltration of tissues in inflammatory processes such as rheumatoid arthritis and alveolitis as well as atherosclerosis. See, *e.g.*, Koch, *J. Clin. Invest.* 90:772-79(1992) and Jones, *J. Immunol.* 149:2147-54 (1992) (incorporated by reference in the specification in paragraph 0006; copies are enclosed for the Examiner's convenience). Additional support can be found, for example, in paragraph 0008 and in Boring *et al.* (1998) *Nature* 394:894-897 (a copy is enclosed for the Examiner's convenience).

Claims 1 and 10 have been amended to specify that the antagonist is presented in a pharmaceutical *composition* (as in canceled claims 3 and 12) rather than a pharmaceutical *carrier*. This provides correct antecedent basis for dependent claims 4 and 13. Support for this amendment can be found on page 9, paragraph 0018.

Priority

The Office Action notes that Application No. 08/182,962 which was filed on January 13, 1994, provides support for administration of MCP-1 receptor antagonists for treatment of disease, but MCP-1 receptor antagonists are allegedly not the same as antibodies. The Examiner alleges that according to the '962 specification (and all other applications from

which the instant application claims priority), antagonist are structurally undefined and are to be identified by performing screening assays. The Office Action concludes that the filing date for the claims drawn to methods of treating disease by administering antibodies receive the benefit of March 1, 2004. The Applicants are urged to point out the specific page and line numbers of the previously filed applications that provide support for the concept of administering antibodies for the treatment of disease if the Applicants disagree with the Examiner's determination of priority.

It is stated for the record that claim 1 (drawn to a method for inhibiting a condition characterized by monocytic infiltrates via administering an MCP-1 receptor antagonist such as an antibody which binds to an MCP-1 receptor polypeptide) should be assigned the priority date of January 13, 1995 in accordance with the parent application 08/446,669, now U.S. Patent No. 6,132,987 (herein the '987 patent). The '987 patent incorporates by reference U.S. Patent No. 5,194,375 which teaches the use of monoclonal antibodies as antagonists for receptor proteins (see column 16, line 59 of the '987 specification). This is evidenced by the instant specification, particularly paragraphs 0084 and 0085. The instant specification teaches the following in paragraphs 0084 and 0085:

[0084] The antagonist is identified by adding an effective amount of an organic compound to the culture medium used to propagate the cells expressing the N-terminal domain of MCP-1 receptor. An effective amount is a concentration sufficient to block the binding of MCP-1 to the receptor domain. The loss in binding of MCP-1 to the receptor may be assayed using various techniques, using intact cells or in solid-phase assays.

[0085] For example, binding assays similar to those described for IL-7 in U.S. Pat. No. 5,194,375 may be used. This type of assay would involve labelling MCP-1 and quantifying the amount of label bound by MCP-1 receptors in the presence and absence of the compound being tested. The label used may, for example, be a radiolabel, *e.g.*, ¹²⁵I or a fluorogenic label.

U.S. Patent No. 5,194,375 which is incorporated by reference in paragraph 0085 states the following in column 19, Example 6, lines 56-66 of its specification:

Preparation of Monoclonal Antibodies to IL-7R

Preparations of purified recombinant IL-7R, for example, human IL-7R, or transfected COS cells expressing high levels of IL-7R are employed to generate monoclonal antibodies against IL-7R using conventional techniques, for example, those disclosed in U.S. Pat. No. 4,411,993. Such antibodies are likely to be useful in interfering with IL-7 binding to IL-7 receptors, for example, in ameliorating toxic or other undesired effects of IL-7, or as components of diagnostic or research assays for IL-7 or soluble IL-7 receptor.

The specification further states in paragraph 18 on page 6:

[0018] A further aspect of the invention therefore are pharmaceutical compositions containing a therapeutically effective amount of an MCP-1 antagonist identified using the assays of this invention. Such MCP-1 antagonist compositions may be employed in therapies for atherosclerosis, cancer and other diseases characterized by monocytic infiltrates. **An additional aspect therefore, the invention includes a method for treating these and/or other diseases and pathological states by administering to a patient a therapeutically effective amount of MCP-1 antagonist, or an active fragment thereof, in a suitable pharmaceutical carrier.** [Emphasis added.]

Thus, it is clear from the specification (and parent specifications) that the Applicants have contemplated the use of antibodies to the MCP-1 receptor for the treatment of diseases that are characterized by monocytic infiltration in 1995. One of skill in the art would understand that the use of antibodies (including monoclonal antibodies) as antagonists to MCP-1 receptor could be easily accomplished by following the teachings of U.S. Patent No. 5,194,375 and the conventional techniques of U.S. Pat. No. 4,411,993. Just as antibodies are useful in

interfering with IL-7 binding to IL-7 receptors, the same is true for antibodies to MCP-1 receptor. Such antibodies would interfere with MCP-1 ligand binding to MCP-1 receptors, thereby acting as antagonists. Thus, the Applicants must not re-teach how to use antibodies as antagonists to a receptor protein if others have already taught so in the prior art. Such antagonists can be used to inhibit monocytic infiltration as taught by the specification.

Rejections Under 35 U.S.C. §112

Claims 1, 4, 7, 9-10, 13, 16 and 18 are rejected under 35 U.S.C. §112, first paragraph, as being allegedly not enabled. The Examiner states that the specification is enabling for a method of administration of anti-MCP-1 receptor antibodies but is allegedly not enabling for inhibition of any condition characterized by monocytic infiltrates. Thus, the Examiner maintained the rejection.

The rejection is respectfully traversed to the extent that it applies to the claims as amended.

In order to advance the case towards allowance, the claims have been further amended. Claim 1 has been amended to specify that the condition characterized by monocytic infiltrates is rheumatoid arthritis, alveolitis or atherosclerosis. Claims 16 and 18 has been canceled.

With respect to the conditions characterized by monocytic infiltrates, the Applicants disclose that MCP-1 has been implicated as an important factor in mediating monocytic infiltration of tissues in inflammatory processes such as rheumatoid arthritis and alveolitis. See, *e.g.*, Koch *et al.*, *J. Clin. Invest.* 90:772-79(1992) and Jones *et al.*, *J. Immunol.* 149:2147-54 (1992). See paragraph 0006 of the specification. Koch *et al.* showed that patients who suffer from rheumatoid arthritis have significantly elevated levels of serum MCP-1 compared to normal serum MCP-1 levels and that MCP-1 itself may function to activate newly recruited mononuclear phagocytes, thereby perpetuating the inflammatory response in the synovial tissue (*e.g.*, MCP-1 has been shown to activate monocytes) (see page 778, Koch *et al.*, 1st column, last paragraph, and 2nd column, first paragraph). Jones *et al.* studied the functional role of MCP-1 in rat models of human disease and showed that MCP-1 plays an important role in

the pathogenesis of IgA immune complex alvelolitis (*i.e.*, IgA immune complex-induced lung injury) in rats. Lung injury is mediated by infiltration of monocytes and macrophages (see page 2148, Jones *et al.*, 1st column, 1st and 2nd paragraphs).

Further, the Applicants have continued their work as evidenced by Boring *et al.* (1998) *Nature* 394:894-897 (a copy is enclosed for the Examiner's convenience). In Boring *et al.*, the Applicants have shown that MCP-1 is linked to the development of atherosclerosis (see abstract). The Applicants generated mice that lack the MCP-1 receptor and crossed them with mice that lack apolipoprotein E (which develop severe atherosclerosis). The Applicants then showed that the selective absence of the MCP-1 receptor decreases the lesion formation quite markedly in apolipoprotein E lacking mice (see abstract). Thus, the Applicants were able to determine that the MCP-1 receptor is a genetic determinant of atherosclerosis *in vivo* and they provided strong evidence for a direct non-cholesterol mediated effect of MCP-1 in macrophage recruitment and atherogenesis (see page 896, second column, second paragraph).

Thus, it is clear from these publications and the teachings of the specification that the blocking of MCP-1 to its MCP-1 receptor interferes with monocytic infiltration which is implicated in a variety of diseases. The courts have repeatedly held that a "patent need not teach, and preferably omits, what is well known in the art" (*Lindemann Maschinenfabrik GMBH v. American Hoist and Derrick Company et al.*, 221 USPQ 481 (Fed. Cir. 1984)).

Further, the Examiner has conceded the following points:

- the amount of experimentation required to make an antibody which binds to SEQ ID NOS: 2 and 4 and inhibits the activity of the receptor is not undue
- there is sufficient guidance to which antibodies can be used in light of the amendments to claim 1
- paragraph 0028 provides support for adequate enablement of conditions characterized by monocytic infiltrates

However, the Office Action indicates that the Applicants have argued that there is adequate support for the limitation "therapeutically effective amount", which appears in claim 10. Herein, the Office Action indicates that the specification does not provide adequate guidance for the skilled artisan to be able to administer a therapeutically effective amount, despite the disclosed range of about 10 ug to about 1 mg per ml per dose administered. Specifically, the Examiner states that this range is very broad and since the units are recited in "per milliliter per dose administered" and there is allegedly no guidance as to how many ml are to be administered, it is only guidance to the concentration but not to the dose, and trial-and-error experimentation would be allegedly required by the skilled artisan.

As indicated in the Applicant's last response, the specification provides a clear definition of *an effective amount* in paragraph 0084, *i.e.*, a concentration sufficient to block the binding of MCP-1 to the receptor domain. This can be measured, for example, by calcium flux (see page 38, paragraph 0130 of the specification; MCP-1 induced a rapid rise in intracellular calcium in 293 cells that were stably transfected with MCP-1RB). The Applicants teach on page 40 (see paragraph 0135) that the hallmark function of MCP-1 is the induction of chemotaxis (*i.e.*, the migration of cells along a concentration gradient) and that modest increases in intracellular calcium are sufficient to initiate and support monocyte chemotaxis. In addition, the skilled artisan is fully aware that the effective amount of an antibody is the concentration sufficient to block the binding of the ligand to its receptor, thereby preventing receptor activation. Since the Examiner has conceded that it does not require undue experimentation to make an antibody which binds to SEQ ID NOS: 2 and 4 in order to inhibit the activity of the receptor, this rejection should be withdrawn.

The Applicants have previously indicated that the use of an antibody is considered a *routine procedure* which was acknowledged by the Examiner (please see the Applicants' last response filed on September 27, 2005, page 11). The effective amount of an antibody that binds to a receptor protein in order to inhibit the activation thereof is part of the standard practice in the art of molecular biology. In addition, the effective amount of the antibody is taught to be about 10 µg/ml to about 1 mg/ml. The Office Action indicates that this range is very broad and that there is allegedly no guidance as to how many milliliters are to be administered. However, such

determination is routine and not undue. The Examiner is reminded that some experimentation is permissible.

"The fact that experimentation may be complex does not necessarily make it undue, if the art typically engages in such experimentation" (*In re Certain Limited -- Charge Cell Culture Microcarriers*, 221 USPQ 1165, 1174 (Int'l Trade Comm'n 1983), *aff'd. sub nom., Massachusetts Institute of Technology v. A. B. Fortia*, 774 F.2d 1104, 227 USPQ 428 (Fed. Cir. 1985)).

In light of the amendments and arguments presented above, the rejection of claims 1, 4, 7, 9-10 and 13 under 35 U.S.C. §112, first paragraph, should be withdrawn.

Claims 1, 4, 7, 9-10, 13, 16 and 18 are rejected under 35 U.S.C. §112, first paragraph for allegedly lacking written description. The Examiner concedes that the term "about 10 µg/ml to about 1 mg/ml" appears in the instant specification and the parent specifications. However, according to the Office Action, the recitation of this term is drawn to the effective amount of uncharacterized antagonist which is to be identified via a screening assay, and the recitation of this term does allegedly not constitute adequate support for administration of antibodies at this concentration.

The rejection is respectfully traversed.

Applicants point the Examiner to paragraphs 0084 and 0085 of the specification, wherein the Applicants discuss how to identify antagonists, including antibodies. As indicated above, the Examiner has conceded that there is *sufficient guidance* to which antibodies can be used in the claimed methods. The effective amount of an antibody that binds to a receptor protein in order to inhibit the activation thereof is a standard practice in the art.

Moreover, MPEP 2163 indicates that the description need only describe in detail that which is new or not conventional (see *Hybritech v. Monoclonal Antibodies*, 802 F.2d at 1384, 231 USPQ at 94). In addition, the courts have held that "disclosure of an antigen fully characterized by its structure, formula, chemical name, physical properties, or deposit in a public depository provides adequate written description of an antibody claimed by its binding affinity to that antigen." (see *Noelle v. Lederman*, 355 F.3d 1343, 1349, 69 USPQ2d 1508, 1514 (Fed. Cir.

2004). Since the structure of the MCP-1 receptor is known and making and using an antibody to a receptor protein is standard in the art, the written description requirement is met and the rejection should be withdrawn.

The Office Action further states that all recitations of administration of monoclonal antibodies are considered new matter because they were allegedly not contemplated in the parent specifications. The Applicants have addressed this issue in detail in the priority section wherein it is shown that antibodies were contemplated in 1995 (please see above).

Claim 18 is rejected under 35 U.S.C. §112, second paragraph for allegedly being indefinite. Claim 18 has been canceled. Thus, this rejection is moot.

Rejection under 35 U.S.C. §102(b)

Claims 1, 7, 9, 10, 13, 16 and 18 are rejected under 35 U.S.C. §102(b), for allegedly being anticipated by LaRosa *et al.*, U.S. Patent No. 6,312,689.

This rejection is respectfully traversed.

Since La Rosa *et al.* was issued on November 6, 2001 and the instant application has a priority date of January 11, 1995, La Rosa *et al.* does not qualify as a reference under 35 U.S.C. §102(b). The issue of priority has been addressed in detail in the priority section above. Claims 16 and 18 have been canceled.

Rejection under 35 U.S.C. §103(a)

Claims 4 and 13 are rejected under 35 U.S.C. §103(a), for allegedly being obvious over LaRosa *et al.*, U.S. Patent No. 6,312,689.

The rejection is respectfully traversed.

As indicated above, La Rosa *et al.* was issued on November 6, 2001 and the instant application has a priority date of January 11, 1995. Thus, La Rosa *et al.* does not qualify as a reference under 35 U.S.C. §103(a) either.

Appl. No. 10/791,166
Amdt. dated March 17, 2006
Reply to Office Action of November 18, 2005

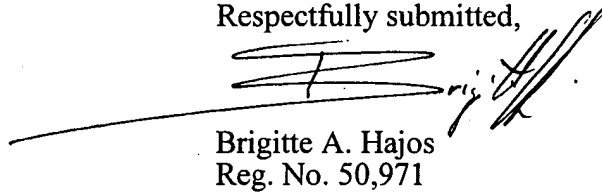
PATENT

CONCLUSION

In view of the foregoing, Applicants believe all claims now pending in this Application are in condition for allowance. The issuance of a formal Notice of Allowance at an early date is respectfully requested.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 415-576-0200.

Respectfully submitted,

A handwritten signature in black ink, appearing to read 'Brigitte A. Hajos', is written over a horizontal line. The signature is stylized with a large 'B' and a long horizontal stroke.

Brigitte A. Hajos
Reg. No. 50,971

TOWNSEND and TOWNSEND and CREW LLP
Two Embarcadero Center, Eighth Floor
San Francisco, California 94111-3834
Tel: 415-576-0200
Fax: 415-576-0300

Attachments: Publications by Jones *et al.* and Koch *et al.* and Boring *et al.*
60699986 v2

Enhanced Production of Monocyte Chemoattractant Protein-1 in Rheumatoid Arthritis

Alisa E. Koch,^{*,†} Steven L. Kunkel,^{†‡} Lisa A. Harlow,^{*,†} Bruce Johnson,^{*,†} Holly L. Evanoff,^{†‡} G. Kenneth Haines,^{†‡} Marie D. Burdick,^{*,†} Richard M. Pope,^{*,†‡} and Robert M. Strieter^{*,†}

Departments of ^{*}Medicine and [†]Pathology, [‡]Northwestern University Medical School, and [§]Veteran's Administration Lakeside Medical Center, Chicago, Illinois 60611; and [¶]The University of Michigan Medical Center, Ann Arbor, Michigan 48109

Abstract

Cells within the synovial tissue may recruit mononuclear phagocytes into the synovial fluid and tissues of arthritic patients. We investigated the production of the chemotactic cytokine monocyte chemoattractant protein-1 (MCP-1) using sera, synovial fluid, synovial tissue, as well as macrophages and fibroblasts isolated from synovial tissues from 80 arthritic patients. MCP-1 levels were significantly higher ($P < 0.05$) in synovial fluid from RA patients (mean 25.5 ± 8.1 ng/ml [SE]) compared to synovial fluid from osteoarthritis (OA) patients (0.92 ± 0.08), or from patients with other arthritides (2.9 ± 1.5). MCP-1 levels in RA sera (8.44 ± 2.33) were significantly greater than MCP-1 in normal sera (0.16 ± 0.06). The quantities of RA synovial fluid IL-8, which is chemotactic for neutrophils and lymphocytes, and MCP-1 were strongly positively correlated ($P < 0.05$). To examine the cellular source of MCP-1, RA synovial tissue macrophages and fibroblasts were isolated. Synovial tissue fibroblasts did not express MCP-1 mRNA, but could be induced to produce MCP-1 by stimulation with either IL-1 β , tumor necrosis factor- α (TNF- α), or LPS. In contrast, unlike normal peripheral blood monocytes or alveolar macrophages, RA synovial tissue macrophages constitutively expressed MCP-1 mRNA and antigen. Immunohistochemical analysis of synovial tissue showed that a significantly greater percentage of RA macrophages ($50 \pm 8\%$) as compared to either OA macrophages (5 ± 2) or normal macrophages (1 ± 0.3) reacted with anti-MCP-1 antibodies. In addition, the synovial lining layer reacted with MCP-1 in both RA and OA synovial tissues. In contrast, only a minority of synovial fibroblasts ($18 \pm 8\%$) from RA synovium were positive for immunolocalization of MCP-1. These results suggest that synovial production of MCP-1 may play an important role in the recruitment of mononuclear phagocytes during inflammation associated with RA and that synovial tissue macrophages are the dominant source of this cytokine. (*J. Clin. Invest.* 1992; 90:772-779.) Key words: mononuclear phagocytes • chemotaxis • cytokine • synovium • synovial fluid

Introduction

Synovial tissue macrophages are important in mediating RA joint destruction, mainly due to their ability to process antigen

and release a variety of cytokines, including IL-1, -6, -8, transforming growth factor- β , tumor necrosis factor- α (TNF- α),¹ and the colony-stimulating factors, macrophage colony-stimulating factor-1 and granulocyte-macrophage colony-stimulating factor (GM-CSF) (1-15). In addition, macrophages mediate the fibroproliferative phase of RA by producing angiogenic activity (16, 17). Furthermore, synovial macrophages may influence synovial fibroblasts to liberate the cytokines IL-6, -8, and GM-CSF, via TNF- α and IL-1, thus creating an operative cytokine network in the joint.

The mechanism by which synovial monocytes are recruited into synovial tissues and fluids has not been fully elucidated. Synovial tissue blood vessel endothelial cells express adhesion molecules like vascular cell adhesion molecule-1, which can mediate the binding of monocytes to blood vessels (18, 19). In addition, it is likely that chemoattractants released by cells in the synovial tissue and fluid recruit mononuclear phagocytes into the joint.

Recently a chemotactic cytokine, termed monocyte chemoattractant protein-1 (MCP-1) has been identified (20-23). This chemotaxin is expressed by a variety of cell types, including leukocytes, smooth muscle cells, endothelial cells, fibroblasts, epithelial cells, and tumor cell lines (20, 24-32). Moreover, this cytokine appears to have selective chemotactic activity for mononuclear phagocytes. The role of this cytokine in the inflamed RA joint has, as yet, not been defined.

In this study, we demonstrated significantly greater MCP-1 levels from synovial fluids of patients with RA as compared with osteoarthritis (OA) or other inflammatory and noninflammatory arthritides. Patients with RA had increased levels of MCP-1 in their serum as compared to normal volunteers. Furthermore, levels of MCP-1 and another chemotaxin, IL-8, were strongly and positively correlated from synovial fluids and sera of RA patients. Isolated RA synovial tissue fibroblasts expressed MCP-1 mRNA and protein in response to either LPS, IL-1 β , or TNF- α stimulation. Isolated RA tissue macrophages constitutively expressed both MCP-1 mRNA and antigenic MCP-1. Finally, we identified MCP-1 by immunohistology in a significantly greater percentage of RA than OA synovial tissue macrophages.

Methods

Reagent preparation. Human recombinant IL-1 β with a sp act of 30 U/ng was a gift from The Upjohn Co. (Kalamazoo, MI). Human recombinant TNF- α with a sp act of 22 U/ng was a gift from Genentech (San Francisco, CA). Human recombinant MCP-1 and IL-8 were

Address correspondence to Alisa E. Koch, M.D., Northwestern University Medical School, Department of Medicine, 303 East Chicago Avenue, Ward Building 3-315, Chicago, IL 60611.

Received for publication 17 January 1992.

The Journal of Clinical Investigation, Inc.
Volume 90, September 1992, 772-779

1. Abbreviations used in this paper: GM-CSF, granulocyte macrophage colony-stimulating factor; MCP-1, monocyte chemoattractant protein-1; OA, osteoarthritis; TNF- α , tumor necrosis factor- α .

purchased from Peptotech, Inc. (Rocky Hill, NJ). Lipopolysaccharide (*Escherichia coli* 0111; B4) was obtained from Sigma Chemical Co. (St. Louis, MO). Polyclonal antihuman IL-8 and MCP-1 were produced by immunization of rabbits with recombinant IL-8 or MCP-1 with CFA. MAb Leu-M5 (anti-CD11c, p150,95, CR4 receptor) detects monocytes and macrophages (Becton Dickinson & Co., Mountain View, CA) as does mAb HAM56 (Enzo Biochem Inc., New York, NY). MAb FVIII detects Factor VIII-related antigen present on endothelial cells (Dakopatts, Carpinteria, CA).

Patient population. Synovial fluids were isolated from patients with either RA, OA, or other arthritides during therapeutic arthrocentesis. Serum specimens were obtained from some of the RA patients as well as from healthy volunteers. Synovial tissue was obtained from patients undergoing total joint replacements who met the American College of Rheumatology criteria for RA or OA (32, 33). Normal synovial tissues were obtained from fresh autopsies. Synovial tissues from these patients were snap frozen in OCT (Miles Laboratories Inc., Elkhart, IN). Alternatively, fresh RA tissues were used for isolation of macrophages or fibroblasts (see below). All specimens were obtained with Institutional Review Board approval. Patient demographic information included synovial fluid leukocyte and differential counts.

Isolation of human RA synovial tissue fibroblasts and macrophages and preparation of conditioned media. Fresh synovial tissues were minced and digested in a solution of dispase, collagenase, and DNase, as previously described (16–18, 34). Synovial fibroblast cells were cultured in RPMI plus 10% FCS containing 1 mM glutamine, 25 mM Hepes, 100 U/ml penicillin, 100 ng/ml streptomycin (Gibco Laboratories, Grand Island, NY), and 1% nonessential amino acids (complete media) in 75-mm tissue culture flasks (Costar Corp., Cambridge, MA). Upon reaching confluence, the cells were passaged by brief trypsinization as previously described (16). The cells were used at passage 4 or older, at which time they were a homogenous population of fibroblastic cells. The cells were plated at a concentration of 8.8×10^4 cells/well in 24-well plates (Costar Corp.) in 1 ml serum-free RPMI. Various concentrations of IL-1 β , TNF- α , or LPS in RPMI were added, and conditioned media harvested.

For isolation of synovial tissue macrophages, the tissues were minced and digested as described above. The resultant single-cell suspensions were fractionated into density-defined subpopulations by isopycnic centrifugation through continuous preformed Percoll gradients (Pharmacia Inc., Piscataway, NJ). Macrophages were enriched by adherence to fibronectin-coated collagen gels and selective trypsinization (incubation with trypsin:EDTA for 5–10 min) (16, 17, 34, 35). Macrophages were harvested from the collagen gels by treatment with clostridial collagenase and found to be $\geq 90\%$ pure, as assessed by Fc receptor-mediated phagocytosis of IgG opsonized sheep red blood cells, esterase staining, and staining with commercial antimacrophage mAbs (16, 17). For preparation of conditioned medium, freshly isolated macrophages were incubated in DME + gentamicin at 1×10^6 cells/ml, and supernatants collected after 24 h.

MCP-1 ELISA. Antigenic MCP-1 was measured using a modification of a double ligand method as previously described (36). In brief, 96-well plates (Nunc, Kamstrup, Denmark) were coated with 50 μ l/well rabbit anti-MCP-1 (3.2 μ g/ml in 0.6 M NaCl, 0.26 M H₃BO₃, and 0.08 N NaOH, pH 9.6) for 16 h at 4°C and then washed in PBS, pH 7.5, 0.05% Tween-20 (wash buffer). Nonspecific binding sites were blocked with 2% BSA in PBS (200 μ l), and the plates incubated for 90 min at 37°C. Plates were rinsed (3 \times) with wash buffer and diluted (neat, 1:5, and 1:10) test sample (50 μ l) in duplicate was added, followed by incubation for 1 h at 37°C. Plates were washed (4 \times) and 50 μ l/well biotinylated rabbit anti-MCP-1 (6 μ g/ml in PBS, pH 7.5, 0.05% Tween-20, 2% FCS) added for 45 min at 37°C. Plates were washed (4 \times), streptavidin-peroxidase conjugate (100 μ g/ml) (Dako patts) added, and the plates were incubated for 30 min at 37°C. The plates were washed (3 \times) and 100 μ l chromogen substrate (0.67 mg/ml orthophenylenediamine dichloride) (Dako patts) added. The plates were incubated at 25°C for 6 min, and the reaction terminated with 50 μ l/well of 3 M H₂SO₄ solution in wash buffer plus 2% FCS. Plates were

read at 490 nm in an ELISA reader. Standards were $\frac{1}{2}$ log dilutions of recombinant MCP-1 from 1,000 ng/ml to 1 pg/ml (50 μ l/well). The ELISA consistently detected MCP-1 concentrations > 0.05 ng/ml.

IL-8 ELISA. Antigenic IL-8 was measured using a modification of a double ligand method as previously described using polyclonal anti-IL-8 (1). The assay was standardized using human recombinant IL-8.

Northern blot analysis. Total cellular RNA was obtained from 2.5×10^6 macrophages or confluent fibroblasts in 100-mm tissue culture dishes using a modification of Chirgwin et al. and Jonas et al. (37–39). Briefly, cells were scraped into a solution containing 25 mM Tris, pH 8.0, 4.2 M guanidine isothiocyanate, 0.5% Sarkosyl, and 0.1 M 2-mercaptoethanol. After homogenization, the suspension was added to a solution containing an equal volume of 100 mM Tris, pH 8.0, 10 mM EDTA, and 1% SDS. The mixture was then extracted with chloroform-phenol and chloroform-isoamyl alcohol. The RNA was alcohol precipitated and the pellet dissolved in diethylpyrocyanate-treated H₂O. Total RNA was separated by Northern analysis using formaldehyde, 1% agarose gels, transblotted onto nitrocellulose, baked, prehybridized, and hybridized with a ³²P-5' end-labeled oligonucleotide probe. A 30-mer oligonucleotide probe was synthesized using the published cDNA sequence for human-derived MCP-1 (20). The probe was complementary to nucleotides 256–285 and had the sequence 5'-TTG-GGT-TTG-CTT-GTC-CAG-GTG-GTC-CAT-GGA-3'. Blots were washed and autoradiographs were quantitated using laser densitometry (Ultrascan XS; LKB Instruments, Inc., Houston, TX). Equivalent amounts of total RNA/gel were assessed by monitoring 28s and 18s rRNA.

Bioassay for chemotactic activity for monocytes. Chemotaxis of monocytes was performed as previously described (24). Normal human mononuclear cells were obtained from peripheral blood by Ficoll-Hypaque density gradient centrifugation. Monocytes were suspended in HBSS with calcium and magnesium (Gibco Laboratories) at 3×10^6 cells/ml with $> 95\%$ viability by trypan blue exclusion. In brief, 150 μ l of synovial fluid or synovial tissue macrophage conditioned medium which was diluted 1:1 with HBSS, 10^{-8} M FMLP (Sigma Chemical Co.), or HBSS alone were placed in duplicate bottom wells of a blind-well chemotaxis chamber. A 5- μ m pore size polyvinylpyrrolidone-free polycarbonate filter (Nuclepore Corp., Pleasanton, CA) was placed in the assembly and 250 μ l of monocyte suspension placed in each of the top wells. Chemotaxis chambers were incubated at 37°C in humidified 95% air/5% CO₂ for 2 h. The filters were removed, fixed in absolute methanol, and stained with 2% toluidine blue (Sigma Chemical Co.). Monocytes that had migrated through to the bottom of the filter were counted in 10 high power fields ($\times 1,000$).

Immunoperoxidase staining. 4- μ m sections of frozen tissues were cut, and immunoperoxidase stained using an avidin-biotin technique (Vector Laboratories, Burlingame, CA) (34, 40–44). Slides, air dried for 2–16 h, were fixed in cold acetone for 20 min. Endogenous peroxidase activity was quenched by incubating the slides for 30 min in 0.3% hydrogen peroxide in methanol. All subsequent incubations were performed for 15 min at 37°C in a moist chamber. The tissue sections were pretreated with 50 μ l diluted normal horse serum (135 μ l horse serum in 10 ml 1% PBS-BSA), incubated with either rabbit anti-human MCP-1, preimmune rabbit serum, mAb Leu-M5, mAb HAM56, or control mAb and washed (2 \times). The slides were incubated with a 1:400 dilution of anti-mouse biotinylated antibody in PBS-BSA, washed (2 \times) with PBS, incubated with avidin/biotinylated horseradish peroxidase complex, and washed with PBS (2 \times). Slides were then stained with diaminobenzidine tetrahydrochloride substrate for 5 min at room temperature, rinsed in tap water for 2 min, counterstained with Harris' hematoxylin, and dipped in saturated lithium carbonate solution for bluing. Serial tissue sections were examined to determine the percentage of each cell type expressing antigenic MCP-1 (18, 34, 43).

Cytospin preparations of isolated RA synovial tissue macrophages were made using 10^4 cells per slide in a cytospin (Shandon I; Shandon Inc., Swickley, PA). Slides were stained using immunohistochemistry as described above.

Statistical analysis. Statistical analysis was performed using analysis of variance (45, 46). *P* values < 0.05 were considered significant.

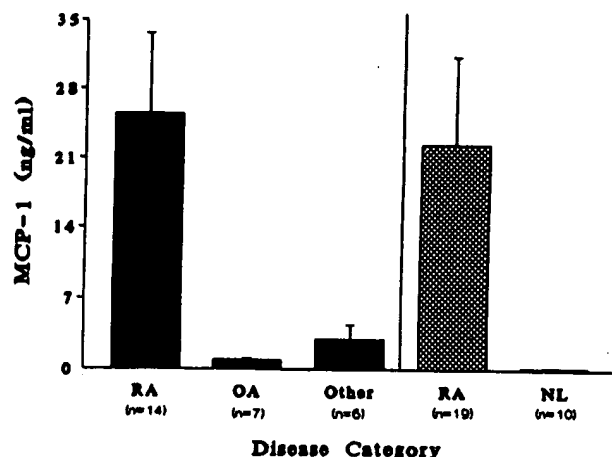


Figure 1. Antigenic MCP-1 levels in synovial fluid from various arthritides as well as RA and normal serum. Results represent the mean \pm SE. Six replicate determinations per patient were performed. N represents the number of patients studied. ■, synovial fluid; ▨, serum.

Results

MCP-1 is elevated in both RA synovial fluid and sera. In initial experiments, MCP-1 was measured by ELISA using samples obtained from 27 patients (Fig. 1). The patients with RA had the greatest levels of MCP-1 (mean 25.5 ± 8.1 ng/ml). OA patients as compared with RA patients had significantly less MCP-1 in their synovial fluids (0.92 ± 0.08 ng/ml, $P < 0.05$). Patients with other forms of inflammatory and noninflammatory arthritis including polymyositis, Reiter's syndrome, chronic lymphocytic leukemia, and mixed connective tissue disease had a mean of 2.9 ± 1.5 ng/ml of synovial fluid MCP-1, which was also significantly less than patients with RA ($P < 0.05$).

To determine whether peripheral blood from patients with RA also contained significant quantities of MCP-1 compared to normal volunteers, serum MCP-1 levels were determined by ELISA using 19 RA samples (Fig. 1). Serum levels of RA MCP-1 ranged from <0.08 ng/ml to 179.2 ng/ml (mean 22.4 ± 8.9). In contrast normal sera obtained from 10 volun-

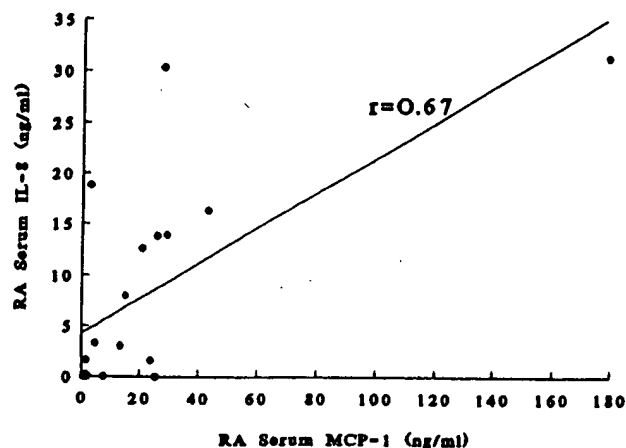


Figure 2. Positive correlation between serum antigenic MCP-1 and IL-8.

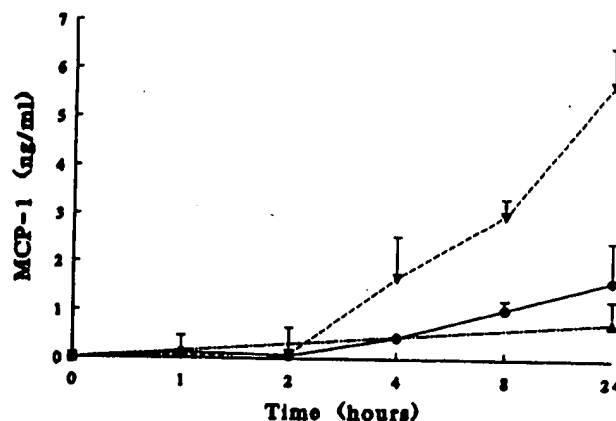
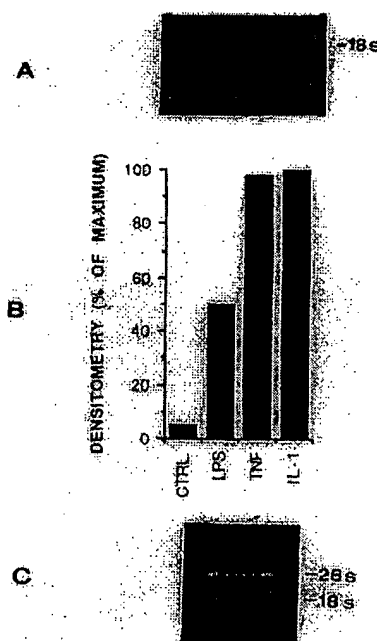


Figure 3. Time-dependent generation of synovial fibroblast MCP-1 by LPS, and IL-1 β stimulation. RA fibroblasts (8.8×10^4 cells/well) were cultured in serum-free RPMI for various time periods. Results represent the mean \pm SE from two patient samples. Six replicates of each sample were assayed. —●—, LPS ($1 \mu\text{g/ml}$); —▲—, IL-1 β (20 ng/ml); —■—, nontreated.

teers contained significantly less MCP-1 (<0.05 to 0.72 ng/ml) (mean 0.16 ± 0.06 , $P < 0.05$). The high levels of MCP-1 present in the RA sera were not attributable to rheumatoid factor in these samples, since there was no correlation between rheumatoid factor positivity (using rabbit IgG) and MCP-1 levels.

Positive correlation between synovial fluid and serum MCP-1 and IL-8 levels. Since cells of the synovial milieu might concomitantly recruit both monocytes and other inflammatory leukocytes, we wished to determine whether those patients who produced large quantities of MCP-1 also produced large quantities of IL-8. ELISA assays were performed on the sera and synovial fluids of arthritic patients to determine the quantities of IL-8 produced. Comparing RA synovial fluid MCP-1 and IL-8 levels, a significant positive correlation ($r = 0.57$, P



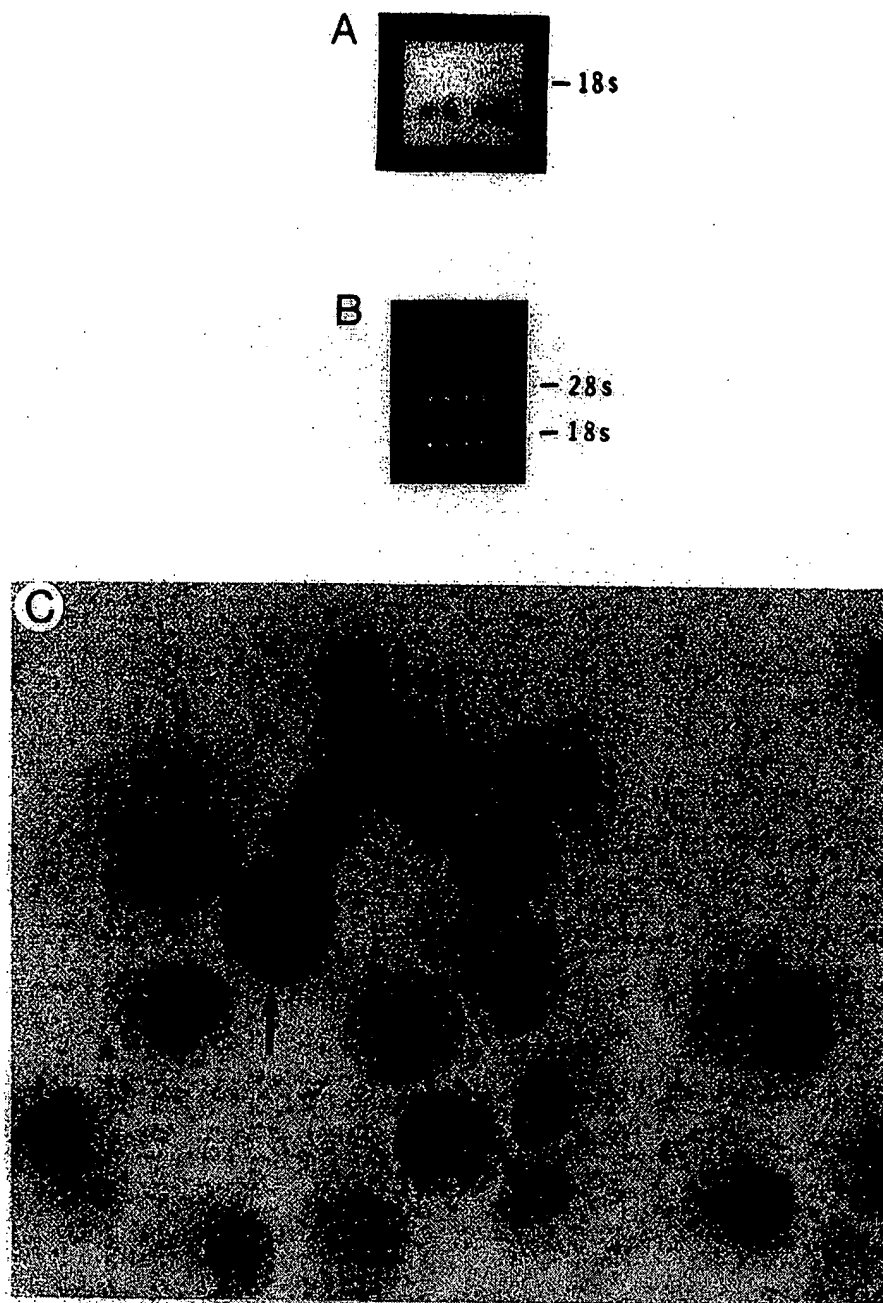


Figure 5. (A) A representative Northern blot showing MCP-1 gene expression from freshly isolated RA synovial tissue macrophages from two patients. (B) 18S and 28S rRNA demonstrating equivalent loading of total RNA in A. (C) Immunoperoxidase stained cytocentrifuge preparation of freshly isolated RA synovial macrophages showing MCP-1 antigen expression (arrows) ($\times 816$).

< 0.05) was found. Similarly, synovial fluid from other arthritides contained MCP-1 and IL-8 levels which were also strongly positively correlated ($r = 0.96$, $P < 0.05$). RA serum MCP-1 and IL-8 levels were correlated, implying a parallel relationship between the amounts of chemotactic cytokines released specific for differing leukocyte populations in this disorder (Fig. 2, $r = 0.67$, $P < 0.05$).

RA synovial fibroblast production of MCP-1. To ascertain whether cellular constituents of the synovial tissue were able to produce MCP-1 in vitro, isolated cell populations from RA synovial tissues were examined. RA fibroblast cells appeared to be a homogenous cellular population of cells with elongated processes. No rounded cells were present. Moreover, RA fibroblasts were esterase negative, and nonreactive with the antima-

crophage mAbs Leu-M5 and HAM 56, excluding the presence of contaminating macrophages. Cytocentrifuge preparations of fibroblasts did not react immunohistochemically with mAb FVIII, excluding the presence of contaminating endothelial cells. LPS induced fibroblast MCP-1 release at concentrations of 100 ng/ml and above. In contrast, as little as 0.02 ng/ml IL-1 β or TNF- α induced fibroblast MCP-1 release. Maximal stimulatory concentrations of IL-1 β and TNF- α were 0.2 ng/ml and 20 ng/ml, respectively. Time-dependent generation of MCP-1 is shown in Fig. 3. LPS-induced fibroblast MCP-1 increased steadily over 24 h as did IL-1 β induced MCP-1. While LPS induced 1.62 ± 0.8 ng/ml (SE) MCP-1, IL-1 β induced 5.7 ± 0.78 ng/ml MCP-1. Nonstimulated fibroblasts released 0.29 ± 0.78 ng/ml MCP-1. Fibroblast MCP-1 gene expression

Table I. Chemotaxis of Monocytes in Response to RA Synovial Fluids and RA Synovial Tissue Macrophage Conditioned Medium Compared to MCP-1 Protein Levels Determined by ELISA Assay

	Mean cells/high power field ($\times 1,000$)	MCP-1 ng/ml
FMLP (10^{-6} M)	44	Not examined
Macrophage-conditioned medium		
Patient number		
1	15	<0.05
2	17	2.3
3	24	0.4
4	29	3.1
5	19	7.4
6	35	1.3
Synovial fluid		
Patient number		
1	23	10.7
2	52	1.4

RA synovial fluids and synovial tissue macrophage-conditioned media were assayed for their ability to induce chemotaxis of monocytes. The results represent analysis of 10 high power fields per sample. Negative control migration in response to HBSS = mean of five cells/high power field. MCP-1 was assayed by ELISA (see Methods).

was absent in nonstimulated RA fibroblasts, but readily inducible upon LPS ($1 \mu\text{g/ml}$), IL- 1β (20 ng/ml), or TNF- α (20 ng/ml) treatment of cells (Fig. 4).

RA synovial fluid and macrophage generated monocyte chemotactic activity. We then determined whether RA synovial fluids and conditioned medium from synovial tissue macrophages produced biologically active chemotactic activity for monocytes. Chemotactic activity for normal human peripheral blood monocytes was detected in the RA synovial fluids examined (Table I). Conditioned medium from nonstimulated macrophages obtained from six patients also contained chemotactic activity for monocytes. Measurable antigenic MCP-1 ranged from <0.05 to 7.4 ng/ml in these samples. The amounts of chemotactic activity for monocytes and antigenic MCP-1 did not correlate, implying the presence of additional chemotactic factors for monocytes in the synovial fluids and in the RA synovial tissue macrophage supernatants.

Synovial tissue macrophage production of MCP-1. To determine if RA synovial tissue macrophages constitutively expressed MCP-1, MCP-1 gene expression was determined using isolated RA synovial tissue macrophages (Fig. 5). In contrast to RA fibroblasts, RA macrophages did not require exogenous stimulation to express MCP-1 mRNA. To confirm macrophage constitutive MCP-1 production, macrophages were freshly isolated from the synovial tissues of RA patients, cytospun, and stained for immunolocalization of MCP-1. Fig. 5 c shows RA synovial tissue macrophage MCP-1 antigen expression.

Immunohistochemical localization of MCP-1 to RA synovial tissue macrophages. To immunolocalize the cells responsible for MCP-1 production in vivo, we examined synovial tis-

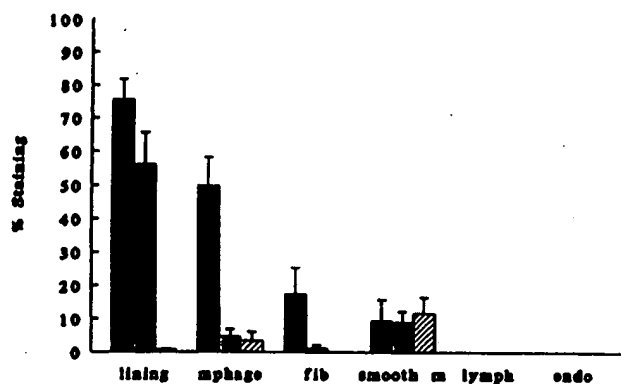


Figure 6. Immunohistochemical demonstration of MCP-1 expression in synovial tissues. *Mphe*, macrophage; *smooth m*, vascular smooth muscle; *fib*, fibroblast; *lymph*, lymphocyte; and *endo*, endothelial cell. ■, RA (n = 13); ■, OA (n = 11); ▨, NL (n = 3).

ues by immunohistochemistry. Synovial tissues from 27 subjects were examined for antigenic MCP-1. The results of immunolocalization are shown in Figs. 6 and 7. In general, the RA synovial tissues had higher inflammatory scores (mean 2.4 ± 0.2) as compared to either the OA tissues (mean 1.5 ± 0.2) or the normal tissues (mean 1.0) (18, 44). The specificity of anti-MCP-1 reactivity with tissues was confirmed by the use of nonimmune serum as well as preadsorption with recombinant human MCP-1. The MCP-1 positive cells were located in the macrophage-rich synovial lining layer, in both RA and OA synovial tissues. MCP-1 immunolocalized to a mean of $76 \pm 6\%$ of the RA lining cells (Fig. 7 A) and $60 \pm 10\%$ of the OA lining cells (Fig. 7 B), but only to $1 \pm 0.3\%$ of the lining cells found in normal synovial tissues (Fig. 7 D) ($P < 0.05$ for either RA or OA compared to normal). RA synovial tissue macrophages located in the subsynovial areas also expressed MCP-1, with $50 \pm 8\%$ of macrophages being MCP-1 positive (Fig. 7 C). In contrast, only $5 \pm 2\%$ of the OA Leu-M5 positive macrophages were MCP-1 positive ($P < 0.05$) (Fig. 7). Similarly, RA macrophage antigenic MCP-1 expression was significantly greater than the expression found in normal synovial tissues ($P < 0.05$).

As compared to the reactivity of the majority of RA macrophages with anti-MCP-1, MCP-1 expression was found in a minority of fibroblasts from both RA (mean of $18 \pm 8\%$) and OA (mean of $9 \pm 3\%$). In addition, a small percentage (mean $9.4 \pm 6.3\%$) of synovial tissue blood vessel smooth muscle cells were MCP-1 positive.

Discussion

MCP-1 is a 76-amino acid basic protein with selective chemotactic activity for mononuclear phagocytes (23, 47). MCP-1 exists in two forms: MCP-1 α , with a mol wt of 13,000, and MCP-1 β , with a mol wt of 15,000 (20, 47). These two forms of MCP-1 are functionally identical, differing only by the extent of posttranslational modification. MCP-1 belongs to a supergene family that includes LD78, ACT-2, RANTES, and I-309 (48).

The role of MCP-1 in the inflamed RA joint may be the recruitment of mononuclear phagocytes. There are a variety of chemotactic factors which may attract mononuclear phagocytes in the RA joint. These include thrombin, C5a, platelet

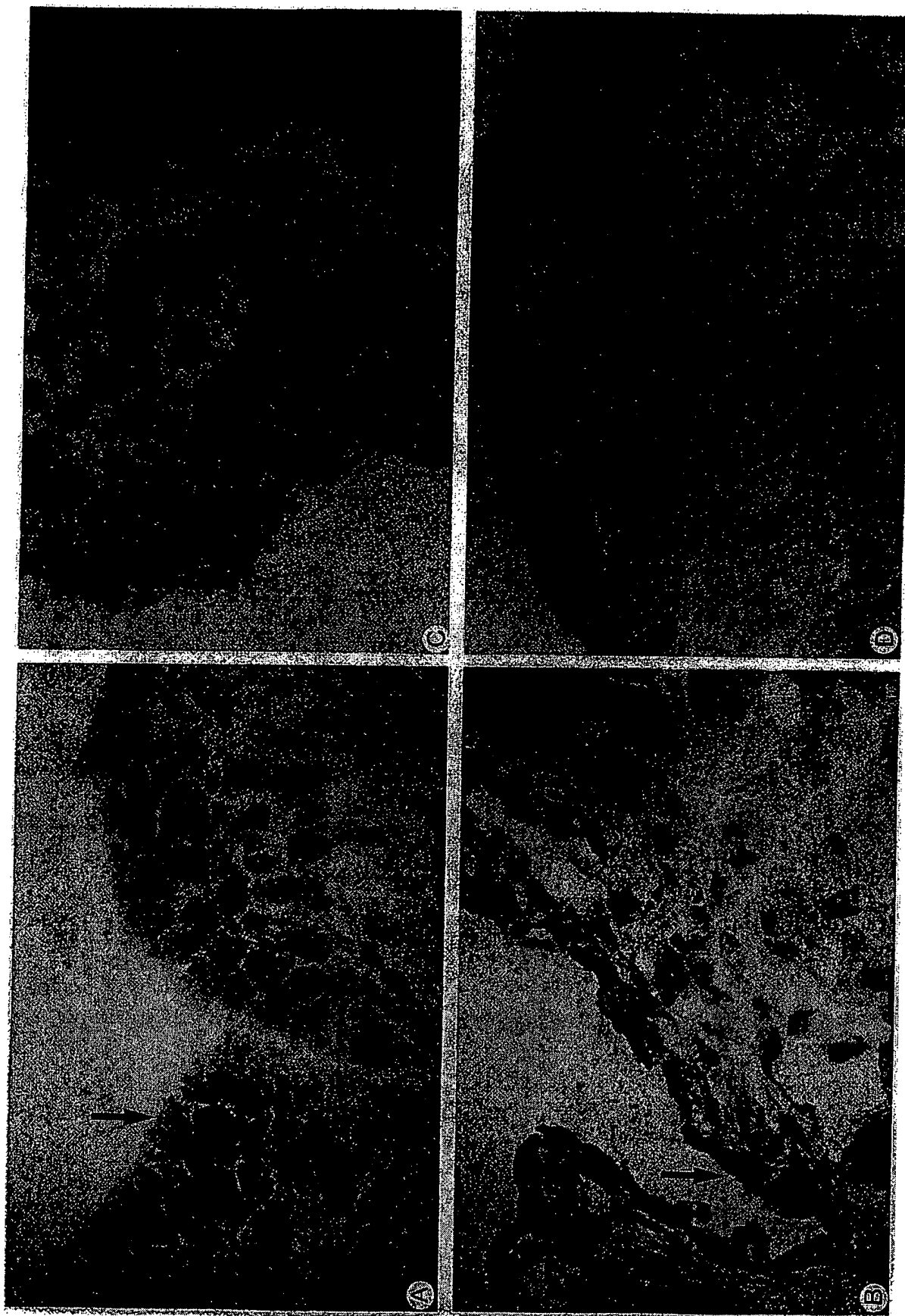


Figure 7. Immunoperoxidase reactivity of anti-MCP-1 with frozen human synovial tissues ($\times 448$). (A) Intense lining layer MCP-1 reactivity in an RA synovial tissue (arrow). (B) Lining layer MCP-1 reactivity in an OA synovial tissue (arrow). (C) Subsynovial macrophage MCP-1 reactivity in an RA synovial tissue (arrows). (D) Normal synovium showing lack of MCP-1 reactivity.

activating factor, leukotriene B₄, lymphocyte-derived chemotactic factor, and the cytokines transforming growth factor-beta, GM-CSF, and platelet-derived growth factor (PDGF) (49, 50). In this study, we demonstrated that the cytokine MCP-1 may also serve as a potent stimulus for recruitment of mononuclear phagocytes in RA.

We examined synovial fluids from patients with various arthritides for MCP-1. MCP-1 levels were significantly elevated in synovial fluids from patients with RA as compared to OA or other arthritides. Moreover, while the serum levels of MCP-1 were 0.16 ± 0.06 ng/ml from normal subjects, MCP-1 RA serum levels were significantly increased (22.4 ± 8.9 ng/ml, $P < 0.05$).

The process of synovial inflammation likely results from the influx of a series of inflammatory cells that release a cascade of inflammatory mediators. We reasoned that synovial fluids and sera containing chemotactic factors for mononuclear phagocytes such as MCP-1 might also contain chemotactic factors for neutrophils and lymphocytes. Indeed, we found a strong positive correlation between MCP-1 and IL-8 in both synovial fluid and serum of patients with RA. These results suggested that the same factors which might upregulate production of MCP-1 also influence the production of IL-8 (1). Hence, we examined whether RA fibroblasts produced MCP-1 in response to the same agonists which upregulated production of synovial fibroblast IL-8. As described by DeMarco et al., who showed that RA synovial fibroblasts expressed MCP-1 mRNA in response to IL-1 and TNF- α , we demonstrated that RA fibroblasts respond to these signals as well as to LPS and produce both MCP-1 mRNA and protein (51). While RA fibroblasts spontaneously produced either nondetectable or low levels of MCP-1, TNF- α , or IL-1 β , and to a lesser extent LPS induced increased levels of fibroblast MCP-1. These same stimuli serve as inducing agents for RA fibroblast IL-8 production. It is likely that in the inflamed RA synovium, macrophages produce IL-1 and TNF- α , both of which act on synovial fibroblasts to stimulate release of IL-8 and MCP-1, which in turn selectively recruit neutrophils, lymphocytes, and mononuclear phagocytes into the joint.

While MCP-1 may be produced by a variety of cells such as blood mononuclear cells, endothelial cells, smooth muscle cells, fibroblasts, epithelial cells, and tumor cell lines, we have identified the synovial tissue macrophage as a major cell producing MCP-1 within the RA joint. Macrophages isolated from RA synovial tissues produced MCP-1 mRNA, antigenic MCP-1, and biologically active chemotactic activity for peripheral blood monocytes. In addition, we immunolocalized MCP-1 to isolated RA synovial tissue macrophages. RA macrophages constitutively produce MCP-1 in greater numbers as compared to either OA or normal synovial tissue macrophages. Interestingly, normal human peripheral blood monocytes or alveolar macrophages do not express MCP-1 mRNA either constitutively or when stimulated with LPS (52). Perhaps, in the inflamed synovial milieu the synovial macrophage assumes a phenotype of chronic activation with subsequent inflammatory cytokine release. Indeed, MCP-1 has been shown to "activate" monocytes, causing cytostatic activity against tumor cell lines, stimulation of the leukocyte respiratory burst, and lysosomal enzyme release (23, 47). It may be that MCP-1 itself may function to activate newly recruited mononuclear phagocytes, hence perpetuating the inflammatory response in the RA synovial tissue.

In summary, synovial fluids from RA patients contain significantly more MCP-1 than fluids from OA patients or patients with other arthritides. RA serum MCP-1 levels were significantly elevated compared to normal serum MCP levels. There was a strong positive correlation between RA synovial fluid or serum levels of MCP-1 and IL-8. Immunohistological examination revealed that a significantly greater proportion of RA than OA or normal macrophages expressed antigenic MCP-1. RA synovial fibroblasts were induced to produce MCP-1 mRNA and protein by IL-1 β , TNF- α , or to a lesser degree LPS. RA macrophages constitutively produced MCP-1 mRNA and protein. These results may help elucidate the mechanism by which mononuclear phagocytes enter both the synovium and synovial fluid in RA.

Acknowledgments

We thank our colleagues, Dr. S. D. Stulberg, C. Schwartz, J. Galante, N. Rana, B. Briggs, and J. Lessard for supplying the synovial tissues. We would also like to thank Drs. Herbert Rubinstein and Frank Schmid for helpful discussions. We also wish to acknowledge the invaluable help of Dr. James Sinacore in performing statistical analysis of the data. We thank Ms. Corinne Washington for expert secretarial assistance.

This work was supported by National Institutes of Health grants AR30692 (A. E. Koch and R. M. Pope), AR41492 (A. E. Koch), HL-02401 (R. M. Strieter), HL-31693 (S. L. Kunkel), an Arthritis Foundation grant (R. M. Pope), an Arthritis Foundation fellowship (A. E. Koch), Veteran's Administration Merit Reviews (A. E. Koch and R. M. Pope), and an American Lung Association research grant. R. M. Strieter is an RJR-Nabisco Research Scholar.

References

- Koch, A. E., S. L. Kunkel, J. C. Burrows, H. L. Evanoff, G. K. Haines, R. M. Pope, and R. M. Strieter. 1991. Synovial tissue macrophage as a source of the chemotactic cytokine IL-8. *J. Immunol.* 147:2187-2195.
- Wood, D. D., E. J. Ihrie, and D. Hamerman. 1985. Release of interleukin-1 from human synovial tissue in vitro. *Arthritis Rheum.* 28:853-862.
- Miyasaka, N., K. Sato, M. Goto, M. Sasano, M. Natsuyama, K. Inoue, and K. Nishioka. 1988. Augmented interleukin-1 production and HLA-DR expression in the synovium of rheumatoid arthritis patients. Possible involvement in joint destruction. *Arthritis Rheum.* 31:480-486.
- Wood, D. D., E. J. Ihrie, C. A. Dinarello, and P. L. Cohen. 1983. Isolation of an interleukin-1-like factor from human joint effusions. *Arthritis Rheum.* 26:975-983.
- Yocum, D. E., L. Esperanza, S. Dubry, J. B. Benjamin, R. Volz, and P. Scuderi. 1989. Characteristics of tumor necrosis factor production in rheumatoid arthritis. *Cell. Immunol.* 122:131-145.
- Husby, G., and R. C. Williams, Jr. 1988. Synovial localization of tumor necrosis factor in patients with rheumatoid arthritis. *J. Autoimmun.* 1:363-371.
- Lipsky, P. E., L. S. Davis, J. J. Cush, and N. Oppenheimer-Marks. 1989. The role of cytokines in the pathogenesis of rheumatoid arthritis. *Springer Semin. Immunopathol.* 11:123-162.
- Arend, W. P., and J. M. Dayer. 1990. Cytokines and cytokine inhibitors or antagonists in rheumatoid arthritis. *Arthritis Rheum.* 33:305-315.
- Dayer, J.-M., B. deRochemonteix, B. Burrus, S. Demczuk, and C. A. Dinarello. 1986. Human recombinant interleukin 1 stimulates collagenase and prostaglandin E₂ production by human synovial cells. *J. Clin. Invest.* 77:645-648.
- Dayer, J.-M., B. Beutler, and A. Cerami. 1985. Cachectin/tumor necrosis factor exerts endocrine, paracrine, and autocrine control of inflammatory responses. *J. Exp. Med.* 162:2163-2168.
- Firestein, G. S., J. M. Alvaro-Gracia, and R. Maki. 1990. Quantitative analysis of cytokine gene expression in rheumatoid arthritis. *J. Immunol.* 144:3347-3353.
- Firestein, G. S. 1991. The immunopathogenesis of rheumatoid arthritis. *Curr. Opin. Rheumatol.* 3:398-406.
- Firestein, G. S., and N. J. Zvaifler. 1990. How important are T cells in chronic rheumatoid synovitis? *Arthritis Rheum.* 33:768-773.
- Alvaro-Gracia, J. M., N. J. Zvaifler, C. B. Brown, K. Kaushansky, and G. S. Firestein. 1991. Analysis of the synovial cells involved in granulocyte-macrophage colony stimulating factor production and gene expression in rheu-

- matoid arthritis and its regulation by IL-1 and tumor necrosis factor- α . *J. Immunol.* 146:3365-3371.
15. Alvaro-Gracia, J. M., N. J. Zvaifler, and G. S. Firestein. 1989. Cytokines in chronic inflammatory arthritis. *J. Exp. Med.* 170:865-875.
16. Koch, A. E., P. J. Polverini, and S. J. Leibovich. 1986. Stimulation of neovascularization by human rheumatoid synovial tissue macrophages. *Arthritis Rheum.* 29:471-479.
17. Koch, A. E., S. J. Leibovich, and P. J. Polverini. 1988. Functional heterogeneity of human rheumatoid synovial tissue macrophages. *J. Rheum.* 15:1058-1063.
18. Koch, A. E., J. C. Burrows, G. K. Haines, T. M. Carlos, J. Harlan, and S. J. Leibovich. 1991. Immunolocalization of leukocyte and endothelial adhesion molecules in human rheumatoid and osteoarthritic synovial tissues. *Lab. Invest.* 64:313-320.
19. Carlos, T., and J. M. Harlan. 1990. Membrane proteins involved phagocyte adherence to endothelium. *Immunol. Rev.* 114:1-28.
20. Yoshimura, T., E. A. Robinson, S. Tanaka, E. Appella, and E. J. Leonard. 1989. Purification and amino acid analysis of two human monocyte chemoattractants produced by phytohemagglutinin-stimulated human blood mononuclear leukocytes. *J. Immunol.* 142:1956-1962.
21. Furutani, Y., H. N. Nomura, M. Notake, Y. Oyama, T. Fukui, M. Yamada, C. G. Larsen, J. J. Oppenheim, and K. Matsushima. 1989. Cloning and sequencing of the cDNA for human monocyte chemoattractant and activating factor (MCAF). *Biochem. Biophys. Res. Commun.* 159:249-255.
22. Yoshimura, T., E. A. Robinson, S. Tanaka, E. Appella, and E. J. Leonard. 1989. Purification and amino acid analysis of two human glioma-derived cytokines. *J. Exp. Med.* 169:1449-1459.
23. Matsushima, K., C. G. Larsen, G. C. DuBois, and J. J. Oppenheim. 1989. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocyte cell line. *J. Exp. Med.* 169:1484-1490.
24. Streiter, R. M., R. Wiggins, S. H. Phan, B. L. Wharram, H. J. Showell, D. G. Remick, S. W. Chensue, and S. L. Kunkel. 1989. Monocyte chemotactic protein gene expression by cytokine-treated human fibroblasts and endothelial cells. *Biochem. Biophys. Res. Commun.* 162:694-700.
25. Sica, A., J. M. Wang, F. Colotta, E. Dejana, A. Mantovani, J. J. Oppenheim, C. G. Larsen, C. O. Zachariae, and K. Matsushima. 1990. Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. *J. Immunol.* 144:3034-3038.
26. Larsen, G. C., C. O. Zachariae, J. J. Oppenheim, and K. Matsushima. 1989. Production of monocyte chemotactic and activating factor (MCAF) by human dermal fibroblasts in response to interleukin-1 or tumor necrosis factor. *Biochem. Biophys. Res. Commun.* 160:1403-1408.
27. Yoshimura, T., and E. J. Leonard. 1990. Secretion by human fibroblasts of monocyte chemoattractant protein-1, the product of gene JE. *J. Immunol.* 144:2377-2383.
28. Graves, D. T., Y. L. Jiang, M. J. Williamson, and A. J. Valente. 1989. Identification of monocyte chemotactic activity produced by malignant cells. *Science (Wash. DC)*. 245:1490-1493.
29. Elner, S. G., R. M. Streiter, V. M. Elner, B. J. Rollins, M. A. Del Monte, and S. L. Kunkel. 1991. Monocyte chemotactic protein gene expression by cytokine treated human retinal pigment epithelial cells. *Lab. Invest.* 64:819-825.
30. Rollins, B. J., P. Stier, T. Ernst, and G. G. Wong. 1989. The human homolog of the JE gene encodes a monocyte secretory protein. *Mol. Cell. Biol.* 9:4687-4695.
31. Rollins, B. J., T. Yoshimura, E. J. Leonard, and J. S. Pober. 1990. Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP-1/JE. *Am. J. Pathol.* 136:1229-1233.
32. Arnett, F. C., S. M. Edworthy, D. A. Bloch, D. J. McShane, J. F. Fries, N. S. Cooper, L. A. Healey, S. R. Kaplan, M. H. Liang, H. S. Luthra, et al. 1988. The American Rheumatism Association 1987 revised criteria for the classification of rheumatoid arthritis. *Arthritis Rheum.* 31:315-324.
33. Altman, R., E. Asch, D. Bloch, G. Bole, D. Borenstein, K. Brandt, W. Christy, T. D. Cooke, R. Greenwald, and M. Hochberg. 1986. Development of criteria for the classification and reporting of osteoarthritis: classification of osteoarthritis of the knee. *Arthritis Rheum.* 29:1039-1049.
34. Koch, A. E., J. C. Burrows, A. Skoutelis, R. Marder, P. Damer, and S. J. Leibovich. 1991. Monoclonal antibodies detecting monocyte/macrophage activation and differentiation antigens and identifying functionally distinct subpopulations of human rheumatoid synovial tissue macrophages. *Am. J. Pathol.* 138:165-173.
35. Koch, A. E., P. J. Polverini, and S. J. Leibovich. 1986. Induction of neovascularization by activated human monocytes. *J. Leukocyte Biol.* 39:233-238.
36. Evanoff, H. L., M. D. Burdick, S. A. Moore, S. L. Kunkel, and R. M. Streiter. 1992. A sensitive ELISA for the detection of human monocyte chemoattractant protein-1 (MCP-1). *Immunol. Invest.* 21:39-45.
37. Standiford, T. J., S. L. Kunkel, M. A. Basha, S. W. Chensue, J. P. Lynch III, G. P. Toews, J. Westwick, and R. M. Strieter. 1990. Interleukin-8 gene expression by a pulmonary epithelial cell line: a model for cytokine networks in the lung. *J. Clin. Invest.* 86:1945-1953.
38. Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry*. 18:5294-5299.
39. Jonas, E., T. D. Sargent, and I. B. Davis. 1985. Epidermal keratin gene expressed in embryos of *xenopus laevis*. *Proc. Natl. Acad. Sci. USA*. 82:5413-5416.
40. Koch, A. E., G. K. Haines, R. Rizzo, J. A. Radosovich, R. M. Pope, P. G. Robinson, and W. H. Pearce. 1990. Human abdominal aortic aneurysms: immunophenotypic analysis suggesting an immune-mediated response. *Am. J. Pathol.* 137:1199-1213.
41. Robinson, P. G., E. W. Eggena, A. E. Koch, and J. A. Radosovich. 1989. A method for immunohistochemical staining with an iron counterstain. *Lab. Med.* 20:569-571.
42. Hsu, S. M., L. Raine, and H. Fanger. 1981. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC with unlabeled antibody (PAP) procedures. *J. Histochem. Cytochem.* 29:577-580.
43. Koch, A. E., J. A. Radosovich, P. G. Robinson, and R. M. Pope. 1990. Distribution of CD45RA+ and CD45RO+ T lymphocyte subsets in rheumatoid arthritis synovial tissue. *J. Clin. Immunol.* 10:192-199.
44. Koch, A. E., J. C. Burrows, R. M. Marder, P. Damer, and S. J. Leibovich. 1990. Reactivity of human tissues with monoclonal antibodies to myeloid activation and differentiation antigens. *Pathobiology*. 58:241-248.
45. Winer, B. J. 1971. Statistical principles in experimental design. McGraw-Hill Inc., New York.
46. Carmer, S. G., and M. R. Swanson. 1973. An evaluation of ten pairwise multiple comparison procedures by Monte Carlo methods. *J. Am. Stat. Assoc.* 68:66-74.
47. Matsushima, K., and J. J. Oppenheim. 1989. Interleukin-8 and MCAF: novel inflammatory cytokines inducible by IL-1 and TNF. *Cytokines*. 1:2-13.
48. Schall, T. J. 1991. Biology of the RANTES/SIS cytokine family. *Cytokines*. 3:165-183.
49. Verghese, M. W., and R. Snyderman. 1989. Chemotaxis and chemotactic factors. In Human Monocytes. M. Zembala and G. L. Asherton, editors. Academic Press Inc., San Diego, CA. 167-175.
50. Harris, E. D. 1988. Pathogenesis of rheumatoid arthritis: a disorder associated with dysfunctional immunoregulation. In Inflammation: Basic Principles and Clinical Correlates. J. I. Gallin, I. M. Goldstein, and R. Snyderman, editors. Raven Press, Ltd., New York. 751-773.
51. DeMarco, D., S. L. Kunkel, R. M. Streiter, M. Basha, and R. B. Zurier. 1991. Interleukin-1 induced gene expression of neutrophil activating protein (interleukin-8) and monocyte chemotactic peptide in human synovial cells. *Biochem. Biophys. Res. Commun.* 174:411-416.
52. Streiter, R. M., S. W. Chensue, M. A. Basha, T. J. Standiford, J. P. Lynch III, M. Baggiolini, and S. L. Kunkel. 1990. Human alveolar macrophage gene expression of interleukin-8 by tumor necrosis factor- α , lipopolysaccharide, and interleukin-1 β . *Am. J. Respir. Cell Mol. Biol.* 2:321-326.

POTENTIAL ROLE OF MONOCYTE CHEMOATTRACTANT PROTEIN 1/JE IN MONOCYTE/MACROPHAGE-DEPENDENT IgA IMMUNE COMPLEX ALVEOLITIS IN THE RAT¹

MICHAEL L. JONES, MICHAEL S. MULLIGAN, CRAIG M. FLORY,
PETER A. WARD, AND JEFFREY S. WARREN²

From the Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109-0602

We have examined the role of monocyte chemoattractant protein 1 (MCP 1) in the pathogenesis of monocyte/macrophage-dependent IgA immune complex alveolitis in the rat. Rat MCP 1 was cloned and expressed in order to facilitate analysis of its function in rat models of human disease. A cDNA library was constructed from rat pulmonary artery endothelial cells stimulated with TNF- α . The cDNA library was screened with synthetic oligonucleotide probes based on the recently published rat MCP 1 cDNA sequence. Among numerous MCP 1-positive clones, four full length (approximately 480 bp) cDNA were rescued, amplified by polymerase chain reaction, and ligated into a pJVTZ baculovirus transfer vector. *Spodoptera frugiperda* insect cells (Sf-21) infected with baculovirus recombinants (*Autographa californica* nuclear polyhedrosis virus) bearing properly oriented MCP 1 cDNA (AcMCP 1) directed the expression of unique peptides of 18, 21, and 23 kDa. Treatment of AcMCP 1-infected Sf-21 cells with tunicamycin resulted in reduced production of the 21- and 23-kDa proteins and an increase in 16- to 18-kDa products, the predicted size range of uncleaved and nonglycosylated rat MCP 1. Denatured and refolded 23-kDa and 21-kDa rat MCP 1 species exhibited dose-dependent monocyte-specific chemotactic activity at concentrations as low as 10^{-10} M whereas the 18-kDa species exhibited negligible activity. Antibodies that react with the 18-kDa, 21-kDa, and 23-kDa MCP 1 bands by Western immunoblot, block rat rMCP 1-directed monocyte chemotaxis, and neutralize monocyte-specific chemotactic activity secreted by TNF-stimulated rat endothelial cells were raised in rabbits immunized with the 23-kDa MCP 1 species. Intravenous administration of anti-MCP 1 antibodies upon initiation of IgA immune complex lung injury resulted in a marked reduction in lung injury as measured by pulmonary vascular permeability, alveolar hemorrhage, and pulmonary monocyte/macrophage recruitment. These data suggest that MCP 1 may play an important role in the pathogenesis of monocyte/

macrophage-dependent IgA immune complex alveolitis in the rat.

Despite the putative importance of IgA in diseases such as IgA nephropathy, Henoch-Schönlein purpura, dermatitis herpetiformis, and some cases of SLE, little is known about the pathogenesis of IgA-triggered tissue injury. We previously described an IgA immune complex-mediated lung injury model in the rat (1, 2). In contrast to various models of IgG immune complex-mediated tissue injury, IgA-induced injury develops fully in neutrophil-depleted rats (2). IgA immune complex lung injury requires an intact C system and is oxygen radical mediated (1, 2). Ultrastructural cytochemical analysis suggests that local production of H₂O₂ by mononuclear phagocytes is an important effector mechanism in IgA lung injury (3). In IgA lung injury monocytes and macrophages are recruited into the parenchyma and can be retrieved in BAL³ fluid during the development of injury. In contrast to IgG immune complex lung injury, in which locally produced TNF mediates neutrophil recruitment, negligible TNF activity can be detected in the BAL fluid of rats with IgA lung injury (4). The mechanisms through which monocytes and macrophages are recruited into the lungs of rats with evolving IgA immune complex-triggered alveolitis are unknown. A potential mediator of monocyte and macrophage recruitment is the monocyte chemoattractant, MCP 1.

MCP 1, known also as monocyte chemotactic and activating factor, is now known to be identical to the murine JE gene product (5). JE was described in 1983 as a gene that is expressed in mouse 3T3 fibroblasts after stimulation with platelet-derived growth factor (6). Although the JE gene product was not identified until later, its cellular sources and the stimuli promoting its expression were studied extensively (reviewed in Reference 7). MCP 1 is secreted either constitutively, or after induction with mitogens, cytokines, or growth factors, in a variety of cell types including lymphocytes, fibroblasts, endothelium, smooth muscle, and several tumor cell lines (reviewed in Reference 7). In vitro studies indicate that MCP 1 can enhance the tumorigenic activity of monocytes against several lines of tumor cells and that it is a potent chemotactic factor for monocytes (7-10).

Although in vitro studies have provided invaluable in-

Received for publication March 31, 1992.

Accepted for publication June 30, 1992.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported, in part, by the National Institutes of Health (HL-40526, HL-48287 and 5T32-HL-07517).

² Address correspondence and reprint requests to Jeffrey S. Warren, M.D., Department of Pathology, The University of Michigan Medical School, 1301 Catherine Street, Box 0602, Ann Arbor, MI 48109-0602.

³ Abbreviations used in this paper: BAL, bronchoalveolar lavage; MCP 1, monocyte chemoattractant protein 1; PCR, polymerase chain reaction; Sf-21, *Spodoptera frugiperda*-21; AcNPV, *Autographa californica* nuclear polyhedrosis virus; AcMCP 1, *Autographa californica* MCP 1; T-TBS, Tween-Tris-buffered saline; moi, multiplicity of infection; DNP-BSA, dinitrophenol-conjugated BSA.

sights into the potential functions of MCP 1, little is known about its role in physiologic or pathologic processes. The *in vitro* activities ascribed to MCP 1 suggest that it may be of fundamental importance as an inducer of the monocyte/macrophage-rich lesions that are characteristic of such pathologic processes as atherosclerosis, chronic inflammation, infiltration of tumors by monocytes, and granulomatous inflammation. Indeed, recent immunohistochemical and *in situ* hybridization analyses have revealed MCP 1 expression within macrophage-rich foci of atherosclerotic lesions in human and rabbit arteries (11, 12). Northern hybridization analyses of whole rat lungs containing glucan-induced granulomas indicate that MCP 1 gene expression is up-regulated during granuloma formation (13). Analyses of the biologic function of MCP 1 in such processes have not yet been carried out. In order to study the functional role of this mediator in rat models of human disease we cloned and expressed rat MCP 1.

In this study, we addressed the pathophysiologic role of MCP 1 in IgA immune complex-induced lung injury in the rat. This is an ideal model because lung injury is mediated by monocytes and macrophages and can be quantitated using several parameters. The data suggest that MCP 1 may play an important role in the pathogenesis of IgA immune complex alveolitis in the rat.

MATERIALS AND METHODS

Materials. *Spodoptera frugiperda* insect cells (Sf-21; B821-01), wild-type baculovirus stock (*Autographa californica* nuclear polyhedrosis virus; AcNPV), β -galactosidase recombinant stock (Ac-pJVETLZ), and AcNPV wild-type DNA were purchased from Invitrogen, San Diego, CA. The pJVETLZ transfer vector, (pBlueBac; a modification of pJVP10) (14), which contains the polyhedrin gene promoter, an *NheI* restriction site, an early transcriptase locus early promoter, and a β -galactosidase-coding region, was kindly provided by Dr. Chris Richardson (Biotechnical Research Institute, Montreal, Canada). Grace's insect cell culture medium was from GIBCO Laboratories, Grand Island, NY. FCS was from GIBCO. Streptomycin, penicillin, and fungizone were from Whittaker Bioproducts, Walkersville, MD. Sea plaque low melting agarose was from FMC Bioproducts, Rockland, ME. Restriction enzymes and other molecular biology materials were obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN; Bethesda Research Laboratories, Gaithersburg, MD; and New England Biolabs, Boston, MA. Synthetic oligodeoxynucleotides were prepared by the DNA Synthesis Facility (D. L. Oxender, Ph.D., Director), University of Michigan, Ann Arbor, MI.

Rat pulmonary artery endothelial cells. Rat pulmonary artery endothelial cells stimulated with human TNF- α were used for con-

Construction and screening of rat cDNA library. Total RNA was isolated by the guanidinium-isothiocyanate method (16) from rat pulmonary artery endothelial cells stimulated with human rTNF- α (200 U/ml) for 4 h. Poly (A) RNA was isolated by oligo(dT)-cellulose chromatography (16). cDNA synthesized by a modification of the Gubler and Hoffman method (17) was used to construct a library in pCDNA II (Invitrogen, San Diego, CA). pCDNA II (bearing an ampicillin-resistance gene) was electroporated into DH1aF' *Escherichia coli* cells and the library amplified on LB + ampicillin (50 μ g/ml) plates. A [³²P]PCR-labeled oligodeoxynucleotide probe (5'-TAC AGC TTC TTT GGG ACA CCT GCT GGT GAT-3'), complementary to positions 160-193 of the rat MCP 1 cDNA sequence provided by T. Yoshimura et al. (18), was used to screen the cDNA library by high density plaque hybridization (16). Hybridization to nitrocellulose filters was carried out at 37°C in 6X SSC, 5X Denhardt's solution, 0.05% sodium pyrophosphate, 1% SDS, 100 μ g/ml salmon sperm DNA, and 10⁶ dpm/ml probe. Filters were washed three times for 5 min with 6X SSC and 0.1% SDS at 37°C, three times for 30 min at 35°C, dried, and exposed to XAR-5 film (Kodak, Rochester, NY) at -70°C with intensifying screens. Appropriately sized MCP 1 cDNA were selected by successful PCR (Taq polymerase; Cetus Immune) amplification of cDNA using primer pairs that contained *NheI* restriction sites and bracketed the 5' and 3' termini of the rat MCP 1 cDNA sequence published by Yoshimura et al. (18). The primer sequences were: 5'-ATC^{NheI} AGC TAG CCG CCA CCA CTA TGC-3' and 3'-CTA AAC CTT ACA CTA^{NheI} CGA TCG GGT GG-5'.

Construction of the baculovirus transfer vector. The resulting MCP 1 cDNA PCR product of one of the selected full length rat MCP 1 clones (clone 2B) was cut with *NheI* according to manufacturer's instructions (New England Biolabs) and the DNA separated in 1% agarose (16). The resulting band was then cut out of the gel and purified using the following two-microfuge tube method. A hole was punched through the bottom of a 500- μ l tube containing angel hair and this tube (to which the DNA sample was added) was placed inside of a larger microfuge tube which was then centrifuged at 14,000 rpm for 5 min. The preparation was extracted in phenol/chloroform/indole acetic acid, the aqueous phase precipitated in cold ethanol, and the resulting DNA quantitated (16). The DNA pellet was then added to ligation mixes (with molar ratios of insert:vector of 0.5:1, 1:1, 2:1, and 4:1), which contained pJVETLZ that had been linearized with *NheI* and phosphatased (16). As a negative control pJVETLZ alone was subjected to the same set of conditions. The ligation products (and controls) were transfected into *E. coli* INV1aF' (DH1 derivative) (Invitrogen) and amplified. MCP 1-positive colonies (see plaque hybridization protocol above) were picked, grown overnight in fungizone ampicillin growth medium, and subjected to DNA extraction (miniprep method) (16). Preparations containing appropriately sized MCP 1 cDNA inserts (\approx 480 bp) were then analyzed for proper insert orientation.

Proper orientation of the rat MCP 1 insert within the pJVETLZ transfer vector was assessed by using the oligonucleotide primer pairs that were complementary to a portion of the insert containing rat MCP 1 (near the 3'-end) and a portion of the transfer vector at a site adjacent to the 5' end of the MCP 1 insert. Using this method only properly oriented rat MCP 1 inserts would be expected to yield an appropriately sized (\approx 480 bp) PCR product. In addition, if insert orientation direction was random, approximately one-half of the insert-bearing vectors would be expected to yield a product. The primers were: "Vector" primer for pJVETLZ;

5'-GCC GGA TTA TTC ATA CCG TC-3' and
[-34 -from *NheI* site - -15]

"Insert" primer for rat MCP 1: 3'-CTA AAC CTT ACA CTA CGA TCG GGT GG-5'.
[451 - 467]

struction of a cDNA library. The cells were extracted from rat pulmonary arteries by perfusion with microcarrier beads, characterized as endothelial, and maintained in culture as previously described by Ginsburg et al. (15). Cells (sixth passage) used for RNA extraction were grown to confluence in 150 cm² T-flasks (Corning Glass, Corning, NY). The day before RNA extraction the culture medium was removed, the monolayers washed twice with HBSS (GIBCO), and the medium replaced with RMPI 1640 (GIBCO) without serum, endothelial cell growth supplement, or heparin. Upon initiation of stimulation, endothelial monolayers were again washed and fresh medium without growth factors was added along with 200 U/ml rTNF- α (Cetus Immune, Emeryville, CA). After 4 h, culture medium was collected and RNA was prepared from the endothelium as described below. For molecular sizing of native rat MCP 1 serum-free conditioned medium was collected after 12 h.

Transfection of *S. frugiperda* cells and isolation of recombinant virus. The MCP 1-bearing transfer vector (AcMCP 1), amplified in *E. coli* INV1aF', was mixed with wild-type AcNPV DNA (2 μ g/1 μ g) and co-precipitated with calcium phosphate (16). This mixture was used to transfect monolayers of Sf-21 cells maintained at a density of 2.0 \times 10⁶ cells/25-mm tissue culture dish. After 4 h the transfection mix was removed and the monolayers washed twice with Grace's medium. After 4 days, serial dilutions (10⁻¹ to 10⁻⁶) of culture supernatant were layered onto 75% confluent monolayers of Sf-21 cells, incubated for 1 h, washed, and overlaid with fresh Grace's medium containing 1% agarose (Sea plaque) and Blue-O-gal substrate (150 μ g/ml; GIBCO). The cultures were maintained for 4 days at 27°C by which time many cells contained occlusion bodies characteristic of the polyhedrin protein. Plaques devoid of occlusion bodies and positive for β -galactosidase expression (blue in the presence of Blue-O-

gal) were purified and amplified by two successive rounds of plaque assays. *S. frugiperda* cells were grown and maintained at 27°C in Grace's insect medium (GIBCO) supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 µg/ml), and fungizone (50 µg/ml) as described by Summers and Smith (19). For amplification of virus, wild-type baculovirus (AcNPV), or recombinant virus (AcMCP 1) was used to infect Sf-21 cells at a moi of 1.0 plaque-forming units/cell. Negative controls included wild-type virus (AcNPV), rMCP 1-negative virus (AcPJVELZ), and noninfected Sf-21 cells.

Production of rat MCP 1 in Sf-21 cells. Recombinant virus stocks were grown from second passage virus in Sf-21 cells to obtain high titers (>10⁸ plaque-forming units/ml). Pilot experiments were conducted to establish the optimal moi and the optimal time after infection to harvest the cells. For high level rat MCP 1 expression, suspension cultures of Sf-21 cells (10⁶ cells/ml) were infected at moi = 1, and harvested 96 h after infection by centrifugation (700 × g). Pelleted cells were washed with Tris/Cl (50 mM; pH 7), 1 mM dithiothreitol and 250 mM sucrose, resuspended in 0.1 vol Tris/Cl (50 mM; pH 7), 1 mM dithiothreitol and 0.5 M NaCl, and then subjected to three freeze-thaw cycles in liquid nitrogen. Culture supernatants, cell pellet lysates (after DNA was sheared by three passes through a 25-gauge needle), and resuspended (0.1 vol; 50 mM Tris/Cl, pH 7, 1 mM dithiothreitol) cell pellets were used in subsequent analyses and preparations.

Characterization of AcMCP 1 expression products. Proteins expressed by AcMCP 1 and negative controls (uninfected Sf-21 cells, Sf-21 cells infected with wild-type baculovirus (AcNPV), and Sf-21 cells transfected with expression vector alone (AcPJVELZ)) were characterized by SDS/PAGE under reducing conditions on 13.5% gels stained with Coomassie blue R or silver (20, 21). Analysis of MCP 1 glycosylation was carried out by SDS/PAGE using Sf-21 cells infected with AcMCP 1 and incubated in the presence or absence of tunicamycin (10 µg/ml). In vitro biologic activity (monocyte and neutrophil chemotaxis) was assessed as described below.

Chemotaxis assays. Monocyte and neutrophil chemotaxis assays were carried out in parallel in 48-well microchemotaxis chambers as described by Falk et al. (22). Cell suspensions (2.25 × 10⁵ cells/well) were added to the top well of the chamber and permitted to migrate through 10 µm polycarbonate membranes (5 µm porosity for monocytes and 3 µm porosity for neutrophils) toward sample-bearing bottom chambers. After a 4-h incubation (37°C, 5% CO₂, humidified) membranes were removed and the nonmigrating cells wiped off. The membranes were then fixed for 10 min in absolute methanol, air dried, and stained for 30 min in 2% toluidine blue. The numbers of cells migrating through the membrane were counted in three random, 10-mm grids at 400×, with the mean ± SEM calculated for triplicate samples. Results are expressed as normalized values representing the percent of maximum FMLP (10⁻⁶ or 10⁻⁸ M; as indicated) positive control, minus negative controls (buffer alone). Peripheral blood human monocytes (80% to 85%) were isolated by centrifugation through Ficoll-Hypaque (Sigma, St. Louis, MO) and Sepacell-MN (Sepratech, Oklahoma City, OK) as described by Vissers et al. (23). Peripheral blood human neutrophils (91% to 96%) were isolated by centrifugation through Ficoll-hypaque as described by Boyum (24).

Protein solubilization and refolding. Freeze-thaw lysates from AcMCP 1-infected Sf-21 cells were subjected to preparative SDS-PAGE (12.5%). Unfixed, unstained 18-kDa, 21-kDa, and 23-kDa MCP 1 bands, and where indicated, a 44-kDa baculoviral protein band, were cut out and electroeluted into 8 M urea plus 10 mM 2-ME. This was dialyzed against 2000 volumes of (1 mM) 2-ME in HBSS (48 h, 4°C).

Rabbit polyclonal anti-MCP 1. Polyclonal rabbit anti-rat MCP 1 was raised against the 23-kDa MCP 1 in 3 kg New Zealand White rabbits (Charles River Laboratories, Wilmington, MA) immunized with MCP 1 (50 µg) emulsified in Hunter's TiterMax (CytRx, Norcross, GA) and boosted after 1 month with MCP 1 (25 µg). Where indicated, the resulting antiserum was affinity purified using a protein A Sepharose column (Sigma). Anti-MCP 1 serum was diluted 1/1 with PBS (100 mM phosphate, pH 8.0, and 150 mM NaCl) and applied slowly to the PBS-washed column. After extensive washing with PBS, the column was stripped with 100 mM sodium acetate buffer, pH 3.0. One milliliter fractions were collected in tubes containing 50 µl of 1 M Tris buffer, pH 8.0. The affinity-purified IgG fraction was then dialyzed against PBS.

Western immunoblot. Sf-21 cell pellet lysates were subjected to SDS/PAGE (12.5%) according to the method of Laemmli (20). The separated proteins were transblotted to nitrocellulose (0.45 µm; Bio-Rad, Richmond, CA) for 1 h at 12 V with a Gene-Screen apparatus (Molecular Scientific, Corvallis, OR). After transfer, the membrane was blocked with T-TBS (20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.05% Tween 20) (v/v) containing 3% BSA, Cohn fraction V, (Sigma) for 2 h at room temperature. After removal of the blocking solution, the

blot was washed with T-TBS (5 min; three times). Primary antibody (affinity-purified rabbit anti-rat MCP 1; 1 mg/ml) was then added at final concentration of 50 µg/ml in T-TBS with 1% BSA (v/v) and incubated for 1 h. The primary antibody was decanted and the membrane washed as described above. After the final wash, secondary antibody (goat anti-rabbit IgG alkaline phosphatase-conjugated antibody (Bio-Rad) was added at a final dilution of 1/3000 in T-TBS with 1% BSA, and incubated for 1 h. The membrane was washed as above and the bands developed by addition of alkaline phosphatase substrate solution (5-bromo-4-chloro-3-indolyl phosphate and nitro-blue tetrazolium in 10 mM Tris; pH 9.5). Rainbow m.w. markers (Amersham, Arlington Heights, IL) were used to estimate m.w.

IgA antibodies and DNP/BSA Ag. Affinity-purified monoclonal IgA (MOPC 315) directed against DNP-BSA was purchased from Sigma. Dinitrophenol-conjugated BSA was prepared according to the method of Eisen (25). The resulting Ag preparation contained an average of 50 DNP groups/BSA molecule.

Rat IgA immune complex-induced alveolitis. Male Long-Evans pathogen-free rats (350 g; Charles River) were used for all studies. Intraperitoneal injections of ketamine (2.5 to 5.0 mg/100 g body weight) and sodium pentobarbital (5 mg/100 g body weight) were given for sedation and anesthesia. IgA immune complex lung injury was induced as previously described (1-4). Antibody solution (IgA anti-DNP/BSA; 400 µg) was instilled into the lungs through a tracheal cannula. In all cases, a final volume of 300 µl was instilled into the lungs. Ag (DNP-BSA; 3.3 mg) was injected i.v. Rats were killed at the indicated times, lung injury was quantitated, and BAL fluid was harvested. Anesthetized rats were exsanguinated through inferior vena caval section before removal of lungs, thus resulting in negligible contamination of lungs with blood. Pulmonary injury was quantitated by permeability, hemorrhage, and morphometric measurements. Permeability indices were calculated by comparing the leakage of ¹²⁵I-labeled bovine γ-globulin from the circulation into the lung to the ¹²⁵I-labeled colloid remaining in 1 ml of blood as previously described (1-4). Hemorrhage indices were calculated by comparing the leakage of ⁵¹Cr-labeled RBC from the circulation into the lung to the ⁵¹Cr-labeled RBC remaining in 1 ml of blood as previously described (1-4). Intravenous anti-MCP 1 or preimmune serum was infused at time zero.

Morphometric analysis of mononuclear phagocyte recruitment and alveolar hemorrhage. Lung samples (1 mm³) were excised from the peripheral aspect of whole lungs that had been fixed in 4% glutaraldehyde under constant pressure inflation (25 cm H₂O) (4). Samples were washed in 0.1 M cacodylate buffer (pH 7.3), embedded in 1 µm thick plastic sections, and stained with toluidine blue. Use of 1-mm³ samples from the peripheral aspects of inflated lung results in sections that contain no large bronchial structures. (Large bronchial structures are defined as muscular airways lined by respiratory epithelium). Plastic embedding (1 µm thick) allows very high morphologic resolution, thus allowing virtually all alveolar and alveolar septal cells to be easily identified. Morphometric analysis of mononuclear phagocyte recruitment and alveolar RBC (hemorrhage) was carried out by a pathologist (J. S. Warren) who was blinded to sample origin. For each condition, five samples were examined. In each sample, 45 to 60 randomly selected 40× microscopic fields (high power field) were analyzed.

Analysis of BAL fluid. Lung lavage contents for cell counts were collected using 5 ml of 37°C, serum-free RPMI 1640 (3, 4). At least 90% of the administered fluid was always recovered, centrifuged (400 × g; 7 min) to separate cells, and the cells were counted. There was no systematic difference in BAL fluid recovery between control and experimental groups of rats.

RESULTS

Rat MCP 1 cDNA cloning. Several studies have revealed that stimulation of human umbilical vein endothelial cells with TNF-α results in the expression of MCP 1 and the secretion of a monocyte-specific chemotactic protein (26-28). Accordingly, we constructed a cDNA library from rat pulmonary artery endothelial cells stimulated with human TNF-α and cloned a full length rat MCP 1 cDNA. The cDNA library, which contained greater than 1.2 × 10⁶ ampicillin-resistant recombinants, was initially screened with an oligodeoxynucleotide probe that was complementary to positions 160-193 of the rat MCP cDNA sequence published by Yoshimura et al. (18). Among numerous MCP 1-positive colonies, four yielded products after PCR in which primers that bracketed the

5' and 3' termini of the published rat MCP 1 cDNA open reading frame were employed (18). Plasmid DNA preparations prepared from these four clones each yielded fragments of approximately 500 to 700 bp after digestion with *Bam*HI and *Spe*I (polylinker sites in the pCDNA II cloning vector). One cDNA clone (clone 2B) was then amplified by PCR using primer pairs that contained *Nhe*I restriction sites and bracketed the 5' and 3' termini of the rat MCP 1 cDNA sequence.

Expression of rat MCP 1 in a baculovirus system. The baculovirus AcNPV is a helper-independent expression vector that has been used successfully to express several eukaryotic genes (reviewed in Reference 29). A rat MCP 1 expression vector was prepared by ligating the full length MCP 1 cDNA clone 2B (at varying insert:vector ratios) into pJVETLZ. Although successful ligations occurred at each insert:vector ratio employed (0.5:1, 1:1, 2:1, 4:1), a 1:1 ratio was used for this construction because it yielded the maximum number of products bearing single insert copies (data not shown). Proper orientation of the rat MCP 1 insert within the pJVETLZ transfer vector was assessed using the PCR with oligonucleotide primer pairs that were complementary to a portion of the insert containing rat MCP 1 (near the 3' end) and a portion of the transfer vector at a site adjacent to the 5' end of the MCP 1 insert. Of seven MCP 1-bearing transfer vectors, four yielded PCR products that indicated proper insert orientation (Fig. 1). Using this method only properly oriented MCP 1 inserts would be expected to yield an appropriately sized (481-bp) PCR product. In addition, if the insert orientation is random, approximately one-half of the insert-bearing vectors would be expected to yield a product.

Recombinant virus (AcMCP 1) was obtained by homologous *in vivo* recombination between wild-type virus (AcNPV) and the MCP 1-bearing transfer vector. Recombinant viruses were purified by two serial passages as described in *Methods*. Sf-21 cells were infected with rAcMCP 1 (third passage), recombinant virus lacking the MCP 1 insert (AcpJVETLZ), or wild-type virus (AcNPV). Uninfected Sf-21 cells were also included as an additional negative control. AcMCP 1 directed the expression of 18-kDa, 21-kDa, and 23-kDa bands that were not present in uninfected Sf-21 cells or in Sf-21 cells infected with either AcNPV or AcpJVETLZ (Fig. 2). The yield of rat

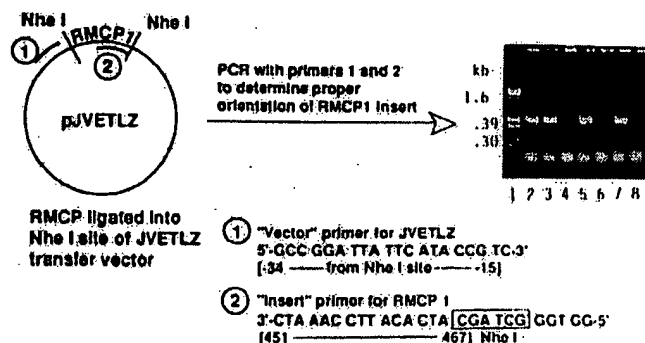


Figure 1. Proper orientation of rat MCP 1 (RMCP 1) in the pJVETLZ transfer vector. Lanes 2, 3, 5, and 7 contain rat MCP 1 insert ligated into pJVETLZ in the proper 5' → 3' orientation. The four properly oriented PCR products (lanes 2, 3, 5, and 7) migrate above the 0.39-kb size marker, at a location consistent with the expected 481-bp PCR product. PCR products were electrophoresed through 0.9% agarose and stained with ethidium bromide.

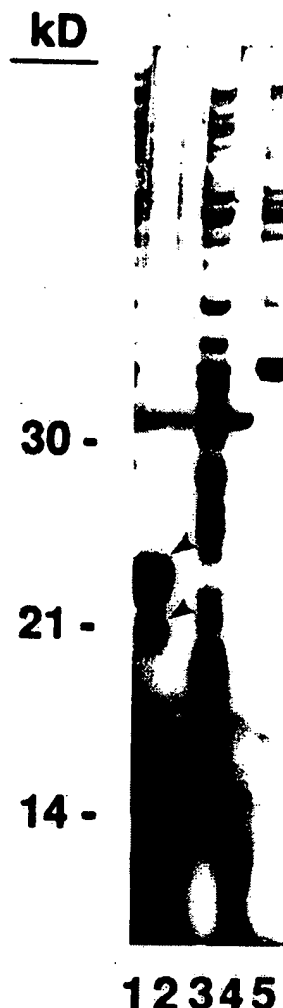


Figure 2. Expression of rat MCP 1 in Sf-21 cells. Sf-21 cells, grown at a density of 2.0×10^6 /25-mm culture dish, were infected with AcMCP 1 (lane 1), wild-type virus (AcNPV) (lanes 2 and 4), no virus (untreated Sf-21 cells) (lane 3), and recombinant virus lacking an MCP 1 insert (AcpJVETLZ) (lane 5). Proteins bands of 18 kDa, 21 kDa, and 23 kDa (arrows) are present only in Sf-21 cells infected with AcMCP 1. These cells were harvested 96 h after infection and lysed in 100 μ l 50-mM Tris/Cl, pH 7, and 1 mM dithiothreitol. After addition of SDS sample buffer, fractions were boiled for 5 min and 20 μ l of each applied to a 13.5% polyacrylamide gel (20). After electrophoresis, the gel was stained with silver according to the method of Wray et al. (21). Lane kd indicates m.w. markers in kilodaltons.

MCP 1 per 2×10^6 starting Sf-21 cells increased as a function of time over 96 h (data not shown). Over the same time period, Sf-21 cells infected with AcpJVETLZ expressed increasing concentrations of β -galactosidase, but no protein in the 18- to 23-kDa range. No new protein bands were observed in Sf-21 cells infected with AcNPV or in uninfected Sf-21 cells at any of the time points examined (data not shown). Essentially all of the stainable rat MCP 1 (18 to 23 kDa protein) was recovered from solubilized Sf-21 cell lysates. The bulk of rat MCP 1 expressed by Sf-21 cells had a m.w. of 23 kDa. These data indicate that Sf-21 cells infected with AcMCP 1 direct the expression of proteins of 18 kDa, 21 kDa, and 23 kDa in a time-dependent manner over 96 h.

Characterization of rat rMCP 1. Based on an open reading frame of 444 bp (18), the predicted m.w. of unprocessed rat MCP 1 is 16.3 to 18.9 kDa. We employed tunicamycin to determine whether the 21-kDa and 23-

kDa protein bands might represent glycosylated species of rat MCP 1. SDS/PAGE analysis of solubilized lysate pellets from Sf-21 cells incubated with tunicamycin revealed several major bands between 16 and 18 kDa and small residual 21-kDa and 23-kDa bands (Fig. 3). This observation suggests that Sf-21 cells infected with AcMCP 1 express variably glycosylated protein species.

Solubilized lysates from Sf-21 cells infected with AcMCP 1 exhibited negligible monocyte or neutrophil chemotactic activity in vitro (data not shown). Accordingly, we sought to solubilize and renature the protein of interest in the hope of producing biologically active rat MCP 1. Cell lysates from AcMCP 1-infected Sf-21 cells (96-h cultures) were subjected to SDS-PAGE. Individual bands (18-kDa, 21-kDa, 23 kDa, and irrelevant 44-kDa baculoviral protein) were cut out, electroeluted, and solubilized in 8 M urea plus 10 mM 2-ME followed by dialysis for 48 h against 2000 volumes of HBSS (4°C) containing 1 mM 2-ME. Renatured cell pellet lysates from AcMCP 1-

infected Sf-21 cells exhibited no neutrophil chemotactic activity (data not shown). The 23-kDa and 21-kDa rat MCP 1 species exhibited dose-dependent, monocyte-specific chemotactic activity, whereas the 18-kDa MCP 1 species exhibited little activity (Fig. 4). As shown in Figure 4, the irrelevant 44-kDa baculoviral protein exhibited no monocyte chemotactic activity.

Characterization of anti-rat MCP 1. Western immunoblot analysis of anti-MCP 1 revealed specific reactivity with the 18-kDa, 21-kDa, and 23-kDa rat MCP 1 species (Fig. 5). Anti-rat MCP 1 serum specifically blocked rat rMCP 1 (23 kDa)-induced monocyte chemotaxis in a dose-dependent manner with 95% blockade of MCP 1 (10^{-6} M) activity with undiluted anti-rat MCP 1 serum (Fig. 6). Equivalent concentrations of preimmune serum exhibited less than 10% blockade of MCP 1-mediated monocyte chemotaxis.

The capacity for anti-rat MCP 1 to neutralize native monocyte chemotactic activity was tested using TNF- α -

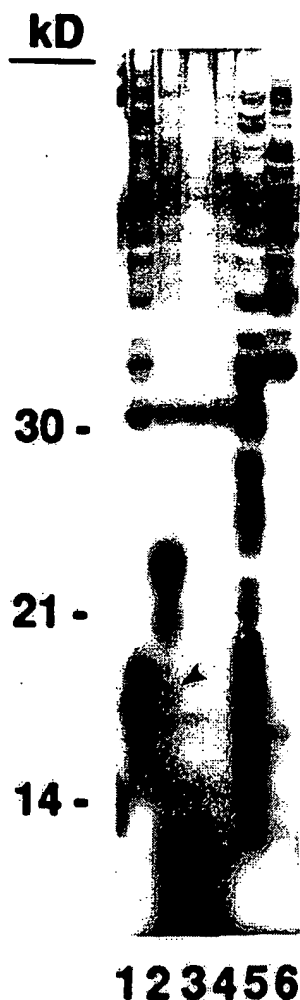


Figure 3. Effect of tunicamycin on rat MCP 1 expression. Addition of tunicamycin to Sf-21 cells infected with AcMCP 1 results in the production of a cluster of protein bands of approximately 16 to 18 kDa. Sf-21 cells, grown at a density of 2.0×10^6 /25-mm culture dish, were infected with AcMCP 1 in the presence of tunicamycin ($10 \mu\text{g}/\text{ml}$) (lane 1). AcMCP 1 in the absence of tunicamycin (lane 2), wild-type virus (AcNPV) (lanes 3 and 4), no virus (uninfected Sf-21 cells) (lane 5), and recombinant virus lacking an MCP 1 insert (Ac β VETLZ) (lane 6). These cells were harvested at 96 h after infection and processed as described in the legend for Figure 2. As in Figure 2, there is a small amount of 18-kDa protein (arrow) produced by Sf-21 cells infected with AcMCP 1 (lane 2).

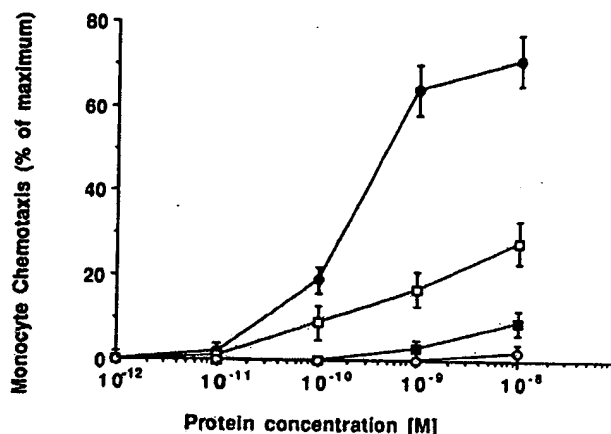


Figure 4. Renatured rat MCP 1 exhibits in vitro monocyte-specific chemotactic activity. The 23-kDa (—●—) and 21-kDa (—□—) MCP 1 species possess potent, dose-dependent monocyte chemotactic activity. The 18-kDa MCP 1 species (—■—) and an irrelevant 44-kDa viral protein (—○—) exhibit little and no monocyte chemotactic activity, respectively. The data (means \pm SEM) are expressed as percentage of maximum monocyte chemotactic response to FMLP (10^{-6} M). The average percentage of input monocytes that migrated in response to FMLP (10^{-6} M) was $27 \pm 4\%$. All samples were assayed in triplicate.

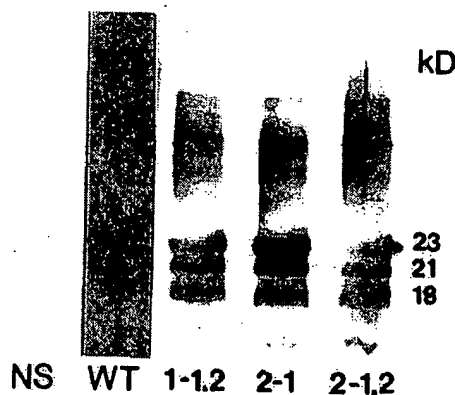


Figure 5. Rabbit polyclonal antibody raised against the 23-kDa rat MCP 1 reacts with the 18-kDa, 21-kDa, and 23-kDa rMCP 1 species. Western immunoblot was carried out as described in *Materials and Methods*. Preimmune serum from rabbit 2 (NS) did not react. Lane WT (wild type) represents reaction between anti-MCP 1 antiserum taken from rabbit 2 and reacted against cell pellet lysates from Sf-21 cells infected with AcNPV. Lanes 1-1.2, 2-1, and 2-1.2 represent anti-MCP 1 antisera obtained from two different rabbits. Antisera were heat inactivated (56°C , 30 min) before application.

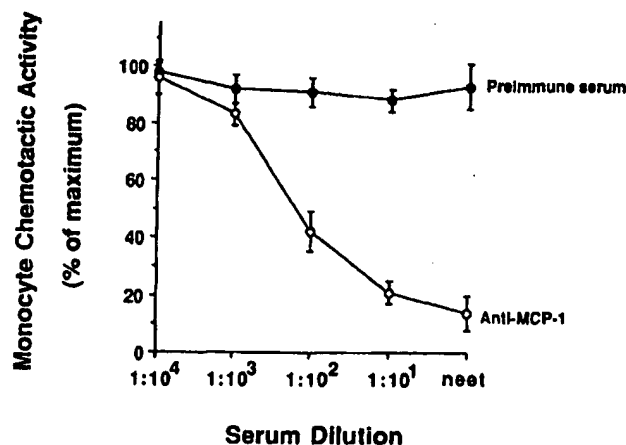


Figure 6. Neutralization of rMCP 1 monocyte chemotactic activity with anti-MCP 1 serum. Data (means \pm SEM; triplicate samples) are expressed as varying dilutions of antiserum vs monocyte chemotactic activity expressed as percentage of maximum response, where 100% is the response to 10^{-8} M MCP 1 (23 kDa). Sample chambers contained the indicated dilutions of heat-inactivated preimmune serum (\bullet) or anti-MCP 1 serum (\circ).

induced monocyte chemotactic activity secreted by rat endothelial cells. Rat pulmonary artery endothelial cells stimulated with human TNF- α expressed increased MCP 1 mRNA levels (data not shown) and secreted an 8- to 30-kDa monocyte-specific chemotactic activity (data not shown), which could be blocked in a dose-dependent manner with anti-rat MCP 1 (62% reduction in monocyte chemotaxis with a 1/100 dilution of anti-MCP 1 serum).

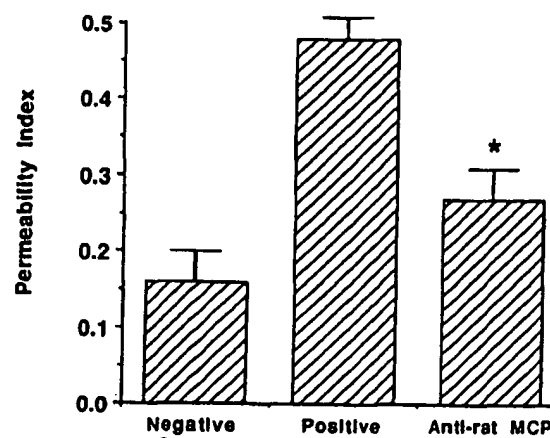
These data indicate that the anti-rat MCP 1 raised against 23-kDa rMCP 1 cross-reacts with the 18- and 21-kDa rMCP 1 species and that it specifically blocks rat rMCP 1 functional activity in vitro. Blockade of the 8- to 30-kDa monocyte-specific chemotactic activity secreted by TNF- α -stimulated rat pulmonary artery endothelial cells indicates that anti-MCP 1 neutralizes native rat MCP 1 activity. It should be noted that anti-MCP 1 had no blocking effect on FMLP (10^{-8} M)-mediated monocyte chemotaxis, zymosan-activated serum-mediated monocyte chemotaxis, or neutrophil chemotaxis (FMLP, 10^{-9} M; zymosan-activated serum) (data not shown).

Role of MCP 1 in pathogenesis of IgA immune complex-triggered alveolitis. Intravenous infusion of anti-MCP 1 antibody upon initiation of IgA immune complex-induced alveolitis resulted in a marked reduction in lung injury as quantified by pulmonary vascular permeability and pulmonary hemorrhage indices (Fig. 7; Table I). Analysis of BAL contents 4 h after anti-MCP 1 infusion revealed a nearly 80% reduction in retrievable mononuclear phagocytes compared with control animals (Table II). These data suggest that MCP 1 is required for the full development of IgA immune complex-induced alveolitis. The morphometric and cell retrieval data indicate that MCP 1 is required for maximum pulmonary monocyte/macrophage recruitment.

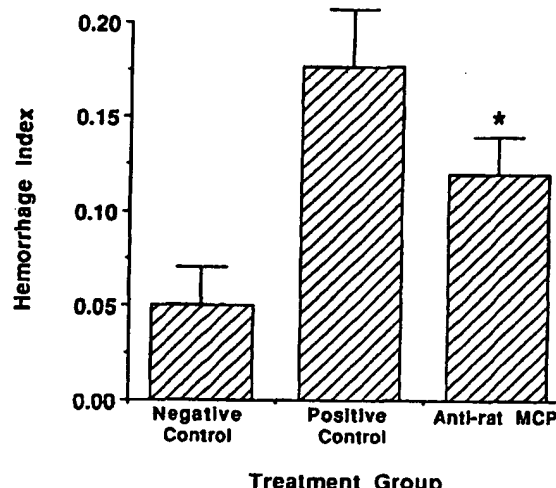
DISCUSSION

Based on in vitro studies that indicate that MCP 1/JE triggers monocyte chemotaxis and activation, and more recently, studies that have revealed either increased MCP 1/JE mRNA levels or immunoreactive MCP 1 monocyte chemotactic activity in atherosclerosis (11, 12), granulomas

A. PERMEABILITY



B. HEMORRHAGE



Treatment Group

Figure 7. Blockade of IgA immune complex lung injury with anti-MCP 1 antibodies. Anti-MCP 1 (0.5 ml) was infused i.v. upon initiation of lung injury. Positive control rats were treated identically but received equivalent quantities of preimmune rabbit serum (0.5 ml) in place of anti-MCP 1. Negative control rats received intratracheal IgA anti-DNP/BSA but no i.v. Ag. Lung injury was quantitated by determining permeability indices (A) and hemorrhage indices (B) 4 h after instillation of anti-DNP/BSA as previously described (1-4). These data represent means \pm SEM of two experiments in which five rats per variable were employed. The data were analyzed by one-way analysis of variance with significance assigned for $p < 0.05$ (37). *Indicates a significant difference vs positive controls.

TABLE I
Morphometric analysis of IgA immune complex alveolitis: effect of anti-MCP 1 antibodies^a

Intervention	Mononuclear Phagocyte Influx		Alveolar Hemorrhage	
	M \pm /40X HPF	p^b	RBC/40X HPF	p^b
A. Preimmune serum (0.5 ml) (positive control)	37 \pm 5		23 \pm 4	
B. Anti-MCP 1 serum (0.5 ml)	18 \pm 3	<0.05 vs A	11 \pm 3	<0.05 vs A
C. None (negative control)	13 \pm 3		4 \pm 1	

^a Immune complex alveolitis and anti-MCP 1 interventions were carried out as described in Figure 7.

^b One-way analysis of variance (37).

(13), and transplanted murine melanomas infiltrated with macrophages (30), it has been suggested that MCP 1/JE mediates monocyte/macrophage-rich pathologic processes. The present study suggests that MCP 1/JE

TABLE II
Effect of anti-MCP 1 on alveolar macrophage retrieval in IgA
alveolitis^a

Treatment	Macrophages Retrieved	% of Total Cells	% Reduction ^b
A. None (negative control)	$4.2 \pm 0.62 \times 10^6$	93%	
B. Preimmune serum (0.5 ml; i.v.)	$9.6 \pm 0.83 \times 10^6$	89%	
C. Anti-MCP 1 serum (0.5 ml; i.v.)	$5.3 \pm 0.47 \times 10^6$	92%	79.7% vs B

^a Immune complex alveolitis and anti-MCP 1 interventions were carried out as described in Figure 7.

^b One-way analysis of variance (37).

may play an important role in the development of monocyte/macrophage-dependent IgA immune complex alveolitis in the rat. Infusion of antibodies directed against rat MCP 1 into rats with developing IgA immune complex-induced alveolitis resulted in a marked decrease in lung injury.

Critical to this study was to clone and express rat MCP 1. We chose the baculovirus/insect cell expression system because it has been successfully used to express other eukaryotic genes as nonfusion proteins (reviewed in Reference 29). Proteins produced in this expression system undergo post-translational processing and have been obtained in relatively large amounts (reviewed in Reference 29). We cloned rat MCP 1 cDNA from rat pulmonary artery endothelium stimulated with TNF- α because others have observed that TNF- α -stimulated human endothelial cells produce MCP 1 (26–28) and because we had previously detected markedly increased levels of rat MCP 1 mRNA in TNF- α -stimulated rat endothelium (M. L. Jones and J. S. Warren, unpublished data). Sf-21 cells infected with AcMCP 1 produced relatively large quantities of biologically inactive rat MCP 1. Solubilization and refolding of MCP 1 contained in Sf-21 cell lysate pellets yielded monocyte-specific chemotactic activity.

Yoshimura et al. recently cloned rat MCP 1 cDNA from Con A-stimulated rat spleen cells (18). The rat MCP 1 cDNA sequence published by Yoshimura et al. is in agreement with the sequence of rat JE genomic DNA reported by Timmers et al. (31). The deduced amino acid sequence of rat MCP 1 bears a strong degree of homology to both human and mouse MCP 1 (32–34). In the present study, SDS/PAGE analysis indicated that the rat MCP 1 species expressed in the baculovirus/insect cell expression system have m.w. of approximately 18 kDa, 21 kDa, and 23 kDa. A shift in the predominant protein species from 23 kDa (and to a lesser extent, 21 kDa), to 16- to 18-kDa species in tunicamycin-treated Sf-21 cell cultures suggests that the two larger species are glycosylated. A m.w. of 16 to 18 kDa agrees with the m.w. of unprocessed rat MCP 1 predicted by its open reading frame (18). Yoshimura et al. reported a 14-kDa monocyte-specific chemotactic activity in the supernatants of rat malignant fibrous histiocytoma cell lines (MFR 11) that express high levels of MCP 1 mRNA (18). A m.w. of 14 kDa approximates the predicted size of mature secreted (cleaved) rat MCP 1 in which the N terminus appears to be the glutamine located at position 24 (18). Amino acid sequence data from mature native human MCP 1 indicates that it starts with a glutamine at position 24. The predicted N-terminal amino acid sequence of unprocessed rat MCP 1 is hydrophobic, typical of a signal peptide, and consistent

with the observation that MCP 1 is a secreted protein.

As reported for various other proteins expressed in baculovirus insect cells (35), rat rMCP 1 was biologically inactive until it was denatured (in 8 M urea and 10 mM 2-ME) and then refolded by dialysis against cold HBSS containing 1 mM 2-ME. The refolded 21-kDa and 23-kDa rat MCP 1 species produced in this study were active in the monocyte chemotaxis assay at concentrations of approximately 35 ng/ml. Although the proportion of rat MCP 1 that is active could not be determined precisely, comparison with the reported potency of native human MCP 1 (optimal activity 10^{-9} M) suggests that only a small proportion of rat MCP 1 (<1%) is active (9, 10). It should be emphasized that this is only an approximation because native human MCP 1 and rat MCP 1 expressed using the baculovirus-insect cell system were not directly compared and because interspecies differences may influence activity quantitated by the monocyte chemotaxis assay. It is presently unknown exactly how polypeptides are produced or stored in a soluble form *in vivo* but several mechanisms may be operative. It is possible that insoluble aggregate formation is favored in insect cells that are overexpressing a foreign protein such as MCP 1. Aggregate formation may be caused by strong hydrophobic and/or ionic interactions among nascent peptides. Alternatively, normal protein folding may require so-called chaperones, proteins that regulate correct self-assembly of nascent peptides without themselves becoming incorporated (reviewed in Reference 36). Little is known about the secretion of native MCP 1 except that it appears to be processed via an N-terminal hydrophobic signal sequence (31–34). Finally, we cannot conclude from this study whether the monocyte chemotactic activity observed in the 21- and 23-kDa species (in contrast to the 18-kDa species) is a function of glycosylation or more efficient refolding.

Provision of MCP 1/JE-specific antibody was paramount to this study. As noted in Figure 5, rabbit polyclonal antibody raised against the 23-kDa rat rMCP 1 species reacted with the 18-kDa, 21-kDa, and 23-kDa baculovirus expression products as would be predicted if these represent variably glycosylated rat MCP 1 species. Anti-rat MCP 1 blocked MCP 1-triggered monocyte chemotaxis in a dose-dependent manner (Fig. 6). Finally, anti-MCP 1 selectively blocked monocyte-specific chemotactic activity secreted by TNF- α -stimulated rat endothelial cells. This observation, supported by the facts that TNF- α -stimulated rat endothelial cells, like human endothelial cells, express increased levels of MCP 1 mRNA (data not shown), that the monocyte-specific chemotactic activity is present in serum-free medium, and that the monocyte chemotactic activity has a m.w. between 8 and 30 kDa, provides compelling evidence that anti-MCP 1 recognizes and neutralizes native rat MCP 1.

The *in vivo* data indicate that MCP 1 is required for full development of IgA immune complex-induced pulmonary vascular leakage and hemorrhage. The lung lavage data (Table II) suggest that MCP 1 may play a role in recruitment of mononuclear phagocytes into the alveolar space. However, it is unclear whether this is a direct effect of local MCP 1 elaboration or a sequel to acute tissue injury *per se*. Attempts to measure MCP 1 activity in BAL fluid and serum were unsuccessful suggesting either that the amounts produced were below the level of detection by

bioassay (10^{-10} M) or that MCP 1 is catabolized, complexed, or otherwise sequestered. It is also possible that MCP 1 is produced chiefly by the endothelium and thus cannot be measured by the assay methods in hand. The relative importance of MCP 1 as a chemotactic factor and as a monocyte/macrophage-activating factor remain to be determined in this model. Despite the issues to be addressed, these data indicate that MCP 1/JE plays an obligate role in the pathogenesis of IgA immune complex alveolitis in the rat.

Acknowledgments. The authors thank Dr. Chris Richardson and Dr. Jorge Vialard for providing the pJVETLZ transfer vector and for discussions regarding the baculovirus expression system, Dr. Teizo Yoshimura for the rat MCP 1 cDNA sequence before it was published, Cynthia Shanteau for manuscript preparation, and Robin Kunkel for figure preparation.

REFERENCES

- Johnson, K. J., B. S. Wilson, G. O. Till, and P. A. Ward. 1984. Acute lung injury in rat caused by immunoglobulin A immune complexes. *J. Clin. Invest.* 74:358.
- Johnson, K. J., P. A. Ward, R. G. Kunkel, and B. S. Wilson. 1986. Mediation of IgA induced lung injury in the rat: role of macrophages and reactive oxygen products. *Lab. Invest.* 54:499.
- Warren, J. S., R. G. Kunkel, R. H. Simon, K. J. Johnson, and P. A. Ward. 1989. Ultrastructural cytochemical analysis of oxygen radical-mediated immunoglobulin A immune complex induced lung injury in the rat. *Lab. Invest.* 60:651.
- Warren, J. S., P. A. Barton, and M. L. Jones. 1991. Contrasting roles for tumor necrosis factor in the pathogenesis of IgA and IgG immune complex lung injury. *Am. J. Pathol.* 138:581.
- Rollins, B. J., P. Stier, T. E. Ernst, and G. G. Wong. 1989. The human homologue of the JE gene encodes a monocyte secretory protein. *Mol. Cell. Biol.* 9:4687.
- Cochran, B. H., A. C. Reffel, and C. D. Stiles. 1983. Molecular cloning of gene sequence regulated by platelet-derived growth factor. *Cell* 33:939.
- Leonard, E. J., and T. Yoshimura. 1990. Human monocyte chemoattractant protein 1 (MCP 1). *Immunol. Today* 11:97.
- Matsushima, K., C. G. Larsen, G. C. DuBois, and J. J. Oppenheim. 1989. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. *J. Exp. Med.* 169:1485.
- Yoshimura, T., E. A. Robinson, S. Tanaka, E. Appella, J. Kuratsu, and E. J. Leonard. 1989. Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. *J. Exp. Med.* 169:1449.
- Yoshimura, T., E. A. Robinson, S. Tanaka, E. Appella, and E. J. Leonard. 1989. Purification and amino acid analysis of two human monocyte chemoattractants produced by phytohemagglutinin-stimulated human blood mononuclear leukocytes. *J. Immunol.* 142:1956.
- Yla-Herttuala, S., B. A. Lipton, M. E. Rosenfield, T. Sarkijöja, T. Yoshimura, E. J. Leonard, J. L. Witztum, and D. Steinberg. 1991. Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA* 88:5252.
- Nelkenna N. A., S. R. Coughlin, D. Gordon, and J. L. Wilcox. 1991. Monocyte chemoattractant protein-1 in human atheromatous plaques. *J. Clin. Invest.* 88:1121.
- Jones, M. L., and J. S. Warren. 1991. Monocyte chemoattractant protein 1 in a rat model of pulmonary granulomatosis. *Lab. Invest.* 66:498.
- Vialard, J., M. Lalvierre, T. Vernet, D. Briedis, G. Alkhatib, D. Henning, D. Levin, and C. Richardson. 1990. Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novel baculovirus vector containing the beta-galactosidase gene. *J. Virol.* 64:37.
- Ginsburg, I., D. F. Gibbs, L. Schuger, K. J. Johnson, V. S. Ryan, P. A. Ward, and J. Varani. 1989. Vascular endothelial cell killing by combinations of membrane-active agents and hydrogen peroxide. *Free Rad. Biol. Med.* 7:369.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Lab., Cold Spring Harbor, NY. pp. 73-82.
- Gubler, U., and B. J. Hoffman. 1983. A simple and very efficient method for generating cDNA libraries. *Gene* 25:263.
- Yoshimura, T., M. Takeya, and K. Takahashi. 1991. Molecular cloning of rat monocyte chemoattractant protein 1 (MCP 1) and its expression in rat spleen cells and tumor cell lines. *Biochem. Biophys. Res. Commun.* 174:504.
- Summers, M. D., and O. E. Smith. 1988. A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agric. Exp. Stn. Bull. No. 1555*.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* 227:680.
- Wray, W., T. Boulikas, V. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Analyt. Biochem.* 118:197.
- Falk, W., R. H. Goodwin, Jr., and E. J. Leonard. 1980. A 48-well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods.* 33:329.
- Visser, M. C. M., S. A. Jester, and J. C. Fantone. Rapid purification of human peripheral blood monocytes by centrifugation through Ficoll-Hypaque and Sepracell-MN. *J. Immunol. Methods* 110:203.
- Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* 21:77.
- Elsen, H. N. 1964. Preparation of purified anti-2,4-dinitrophenol antibodies. *Methods Med. Res.* 10:94.
- Strieter, R. M., R. Wiggins, S. Phan, B. L. Wharram, H. J. Showell, D. G. Remick, S. W. Chensue, and S. L. Kunkel. 1989. Monocyte chemotactic protein gene expression by cytokine-treated human fibroblasts and endothelial cells. *Biochem. Biophys. Res. Commun.* 162:694.
- Dixit, V. M., S. Green, V. Sarma, L. B. Holzman, F. W. Wolf, K. O. O'Rourke, P. A. Ward, E. V. Prochownik, and R. M. Marks. 1990. Tumor necrosis factor- α induction of novel gene products in human endothelial cells including a macrophage-specific chemotaxin. *J. Biol. Chem.* 265:2973.
- Rollins, B., T. Yoshimura, E. J. Leonard, and J. S. Pober. 1990. Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP 1/JE. *Am. J. Pathol.* 136:1229.
- Luckow, V. A., and M. D. Summers. 1988. Trends in the development of baculovirus expression vectors. *Bio/Technology* 6:47.
- Botlaiz, B., S. Walter, D. Govani, F. Colotta, and A. Mantovani. 1992. Monocyte chemotactic cytokine gene transfer modulates macrophage infiltration, growth, and susceptibility to IL-2 therapy of a murine melanoma. *J. Immunol.* 148:1280.
- Timmers, H. T. M., G. J. Pronk, J. L. Box, and A. J. van der Eb. 1990. Analysis of the rat JE gene promoter identities on AP-1 binding site essential for basal expression but not for TPA induction. *Nucleic Acids Res.* 18:23.
- Robinson, E. A., T. Yoshimura, E. J. Leonard, S. Tanaka, P. R. Griffin, J. Shabanowitz, D. F. Hunt, and E. Appella. 1989. Complete amino acid sequence of a human monocyte chemoattractant, a putative mediator of cellular immune reactions. *Proc. Natl. Acad. Sci. USA* 86:1850.
- Yoshimura, T., N. Yuhki, S. K. Moore, E. Appella, M. I. Lerman, and E. J. Leonard. 1989. Human monocyte chemoattractant protein-1 (MCP-1): full length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. *FEBS Lett.* 244:487.
- Rollins, B. J., E. D. Morrison, and C. D. Stiles. 1988. Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. *Proc. Natl. Acad. Sci. USA* 85:3738.
- Berndt, N., and P. T. W. Cohen. 1990. Renaturation of protein phosphatase 1 expressed at high levels in insect cells using a baculovirus vector. *Eur. J. Biochem.* 190:291.
- Ellis, R. J., and S. M. Hemmingsen. 1989. Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. *Trends Biochem. Sci.* 14:339.
- Linton, M., and P. S. Gallo, Jr. 1975. *The Practical Statistician: Simplified Handbook of Statistics*. Brooks-Cole Publishing Co., Monterey, CA. pp. 136-218.

strengthening of neuronal connections during the detection and storage of new information by the hippocampus. □

Methods

Electrode implantation and electrophysiology. Experiments were carried out on freely behaving male Wistar rats (200–300 g) that had electrodes implanted under pentobarbitone (60 mg kg⁻¹) anaesthesia. Recordings of field EPSPs were made from the CA1 stratum radiatum of the hippocampus in response to ipsilateral stimulation of the Schaffer collateral/commissural pathway using techniques similar to those described^{24,27}. Animals recovered at least 14 days before the start of the experiment. Test EPSPs were evoked at a frequency of 0.033 Hz and at a stimulation intensity adjusted to give an EPSP amplitude of 50% of maximum. The high-frequency stimulation protocol for inducing LTP consisted of 10 trains of 20 stimuli, interstimulus interval 5 ms (200 Hz), intertrain interval, 2 s. Repeated stimulation with this protocol fails to increase the magnitude of LTP, indicating that it is almost at saturation for the group of synapses under observation²⁵. LTP was measured as mean \pm s.e.m.% of baseline EPSP amplitude recorded over at least a 20-min baseline period. The EEG was simultaneously monitored (from the hippocampal recording electrode) during all experiments so as to ensure that no abnormal activity was evoked by the conditioning stimulation and to monitor hippocampal theta EEG activity. The spectral power of the EEG was measured after fast Fourier transformation of sweeps of 1.2 s duration. Dual pathway experiments, with two independent ipsilateral stimulation inputs to the same recording electrode, were carried out for most experiments. Lack of paired-pulse interaction with responses evoked in the test pathway was used as a criterion of independence.

Recording apparatus and novelty exploration. To allow free exploration without extensive locomotion (which affects brain temperature and field potential measures of synaptic transmission^{4,28,29}), the recording boxes were relatively small (0.07 or 0.08 m²). Under these conditions, only very transient (<10 min) and small changes (<1 °C) in brain temperature were observed on entering the novel environment.

Experiments were carried out in a well lit (~750 lux, fluorescent lighting) room. The familiar box was made of clear perspex, whereas the novel box was made of Perspex covered with a thin sheet of plastic which acted as a red filter (>600 nm, filter factor ~3 \times). The boxes in the first study had different shapes (34 \times 24 \times 24 cm for the familiar, versus 32 \times 21 \times 20 cm for the novel box). In the other studies, an opaque barrier that separated the familiar and novel environments was removed at 90 min and was closed 20 min later when the animal was in the novel box. To make the novel environment more distinct, the bedding was also different (none in the familiar, versus wood shavings in the novel box). The bedding was changed between rats but was not changed after each trial for a given rat. Behavioural evidence that the animals acquired information about the new environment was provided by the observation that the animals explored less on re-exposure to the novel box on consecutive days (for example, 24 \pm 6 versus 14 \pm 4 transitions between the familiar and novel boxes in the first 20 min on the first and third day, respectively; P < 0.05). Entry into the novel box did not elicit any observable stress responses either hormonally (plasma corticosterone, 5.2 \pm 1.2 versus 3 \pm 0.8 μ g dl⁻¹ in familiar box, measured by HPLC; n = 4)²⁷ or behaviourally (no evidence of behavioural freezing, piloerection or defecation typical of stress). The animals were housed individually in their home cage between recording sessions. Statistical comparisons were made by using Friedman two-way analysis of variance by ranks and Mann–Whitney U -test where appropriate.

Received 20 March; accepted 9 June 1998.

- Martinez, J. & Derrick, B. Long-term potentiation and learning. *Annu. Rev. Psychol.* 47, 173–203 (1996).
- Jeffery, K. LTP and spatial learning—Where to next? *Hippocampus* 7, 95–110 (1997).
- Morris, R. G. M. & Frey, U. Hippocampal synaptic plasticity: role in spatial learning or the automatic recording of attended experience? *Phil. Trans. R. Soc. Lond. B* 352, 1489–1503 (1997).
- Hargreaves, E. L., Cain, D. P. & Vandervolf, C. Learning and behavioral long-term potentiation: importance of controlling for motor activity. *J. Neurosci.* 10, 1472–1478 (1990).
- Brickson, C. A., McNaughton, B. L. & Barnes, C. A. Comparison of long-term enhancement and short-term exploratory modulation of perforant path synaptic transmission. *Brain Res.* 615, 275–280 (1993).
- Moser, E., Moser, M.-B. & Andersen, P. Potentiation of dentate synapses initiated by exploratory learning in rats: dissociation from brain temperature, motor activity, and arousal. *Learning Memory* 1, 55–73 (1994).
- Abraham, W. C. & Bear, M. F. Metaplasticity: the plasticity of synaptic plasticity. *Trends Neurosci.* 19, 126–130 (1996).

- O'Keefe, J. & Nadel, L. *The Hippocampus as a Cognitive Map* (Clarendon, Oxford, 1978).
- Eichenbaum, H. Is the rodent hippocampus just for 'place'? *Curr. Opin. Neurobiol.* 6, 187–195 (1996).
- Green, J. & Arduini, A. Hippocampal electrical activity in arousal. *J. Neurophysiol.* 17, 553–557 (1954).
- Bland, B. The physiology and pharmacology of hippocampal formation theta rhythms. *Prog. Neurobiol.* 26, 1–54 (1986).
- Buzsáki, G. Two stage model of memory trace formation: a role for noisy brain states. *Neuroscience* 31, 551–570 (1989).
- Huang, Y.-Y., Nguyen, P., Abel, T. & Kandel, E. Long-lasting forms of synaptic potentiation in the mammalian hippocampus. *Learning Memory* 3, 74–85 (1996).
- Süßbül, U. & Lynch, G. Stable depression of potentiated synaptic responses in the hippocampus with 1–5 Hz stimulation. *Brain Res.* 513, 113–118 (1990).
- Doyle, C. A., Cullen, W. K., Rowan, M. J. & Anwyl, R. Low-frequency stimulation induces homosynaptic depotentiation but not long-term depression of synaptic transmission in the adult anesthetized and awake rat hippocampus *in vivo*. *J. Neuroscience* 17, 75–85 (1997).
- Manahan-Vaughan, D. Group 1 and 2 metabotropic glutamate receptors play differential roles in hippocampal long-term depression and long-term potentiation in freely moving rats. *J. Neurosci.* 17, 3303–3311 (1997).
- Errington, M. L. et al. Stimulation at 1–5 Hz does not produce long-term depression or depotentiation in the hippocampus of the adult rat *in vivo*. *J. Neurophysiol.* 74, 1793–1799 (1995).
- Huerta, P. & Lisman, J. Bidirectional synaptic plasticity induced by a single burst during cholinergic theta oscillation in CA1 *in vitro*. *Neuron* 15, 1053–1063 (1995).
- Stäubli, U. & Chun, D. Factors regulating the reversibility of long-term potentiation. *J. Neurosci.* 16, 853–860 (1996).
- Parkin, A. J. Human memory: novelty, association and the brain. *Curr. Biol.* 7, R768–R769 (1997).
- Grunwald, T., Lehnertz, K., Heinze, H. J., Helmstaedter, C. & Elger, C. E. Verbal novelty detection within the human hippocampus proper. *Proc. Natl. Acad. Sci. USA* 95, 3193–3197 (1998).
- Honey, R. C., Watt, A. & Good, M. Hippocampal lesions disrupt an associative mismatch process. *J. Neurosci.* 18, 2226–2230 (1998).
- Gluck, M. & Myers, C. Psychobiological models of hippocampal function in learning and memory. *Annu. Rev. Psychol.* 48, 481–514 (1997).
- Miller, K. Synaptic economics: competition and cooperation in synaptic plasticity. *Neuron* 17, 371–374 (1996).
- Dudai, Y. Consolidation: fragility on the road to the engram. *Neuron* 17, 367–370 (1996).
- Doyle, C., Hobeles, C., Rowan, M. J. & Anwyl, R. The selective neuronal NO synthase inhibitor 7-nitro-indazole blocks both long-term potentiation and depotentiation of field EPSPs in rat hippocampal CA1 *in vivo*. *J. Neurosci.* 16, 418–424 (1996).
- Xu, L., Anwyl, R. & Rowan, M. J. Behavioural stress facilitates the induction of long-term depression in the hippocampus. *Nature* 387, 497–500 (1997).
- Leung, L. Behavior-dependent evoked potentials in the hippocampal CA1 region of the rat 1. Correlation with behavior and EEG. *Brain Res.* 198, 95–117 (1980).
- Moser, E., Mathiesen, I. & Andersen, P. Association between brain temperature and dentate field potentials in exploring and swimming rats. *Science* 259, 1324–1326 (1995).

Acknowledgements. This research was supported by the Health Research Board of Ireland, the European Union DGXXI and the Wellcome Trust. We thank W. K. Cullen and J. Wu for assistance.

Correspondence and requests for material should be addressed to M.J.R. (e-mail: mrowan@mail.tcd.ie).

Decreased lesion formation in CCR2^{-/-} mice reveals a role for chemokines in the initiation of atherosclerosis

Landin Borjesson[†], Jennifa Gosling^{*}, Michael Cleary^{*} & Israel F. Charo^{††}

^{*} Gladstone Institute of Cardiovascular Disease, San Francisco, California 94141, USA

[†] Cardiovascular Research Institute, University of California, San Francisco, California 94110, USA

^{††} Department of Medicine and the Daiichi Research Center, University of California, San Francisco, California 94110, USA

Chemokines are proinflammatory cytokines that function in leukocyte chemoattraction and activation and have recently been shown to block the HIV-1 infection of target cells through interactions with chemokine receptors^{1,2}. In addition to their function in viral disease, chemokines have been implicated in the pathogenesis of atherosclerosis. Expression of the CC chemokine monocyte chemoattractant protein-1 (MCP-1) is upregulated in human atherosclerotic plaques^{3,4}, in arteries of primates on a hypercholesterolaemic diet⁵ and in vascular endothelial and smooth muscle cells exposed to minimally modified lipids^{6,7}. To determine whether MCP-1 is causally related to the development of atherosclerosis, we generated mice that lack CCR2, the receptor for MCP-1 (ref. 7), and crossed them with apolipoprotein (apo) E-

null mice¹⁻¹⁰ which develop severe atherosclerosis. Here we show that the selective absence of CCR2 decreases lesion formation markedly in apoE^{-/-} mice but has no effect on plasma lipid or lipoprotein concentrations. These data reveal a role for MCP-1 in the development of early atherosclerotic lesions and suggest that upregulation of this chemokine by minimally oxidized lipids is an important link between hyperlipidaemia and fatty streak formation.

The arterial fatty streak is composed of lipid-laden macrophages (foam cells) and is the precursor of more complex and dangerous lesions. The molecular signals that initiate monocyte/macrophage recruitment to the vessel wall are, however, unknown. We stained aortic root sections from mice on the Western diet¹¹ with MOMA-2, a macrophage-specific antibody¹². Macrophages were abundant in the subendothelial space of CCR2^{+/+}, apoE^{-/-} animals fed the high-fat diet for as little as 5 weeks, and constituted most of the cells in the lesion (Fig. 1a). In contrast, markedly fewer macrophages were present in the aortas of CCR2^{-/-}, apoE^{-/-} mice (Fig. 1b). Quantitative analysis revealed significantly less MOMA-2-positive staining in CCR2^{-/-} mice than in CCR2^{+/+} mice (Fig. 1c). These data indicate that activation of CCR2 was important in recruitment of mono-

cytes/macrophages into the vessel wall, the earliest recognizable sign of atherosclerosis. Staining with Oil Red O confirmed the significant decrease in lesion area in CCR2^{-/-} mice fed the Western diet for 5 weeks (Fig. 2). Lesion size in the CCR2^{+/+} mice was intermediate between the lesion sizes of wild-type and CCR2^{-/-} mice, suggesting a gene dosage effect (Fig. 2). After 9 weeks on the diet, there was no difference between the CCR2^{+/+} and wild-type mice, and both had significantly larger lesions than the CCR2^{-/-} mice. The reduction in lesion size was observed throughout the aortic root and was most pronounced in the valve leaflet region, where wild-type mice developed the most severe lesions (not shown). After 13 weeks on the diet, CCR2^{-/-} mice still had significantly smaller lesions than CCR2^{+/+} mice (Fig. 2). Thus, lesion development was decreased at all time points examined. In addition, at both 9 and 13 weeks, the lesions were less complex in the CCR2^{-/-} mice (not shown). These data suggest that the decreased recruitment of macrophages observed at 5 weeks reduced lesion size at the later time points.

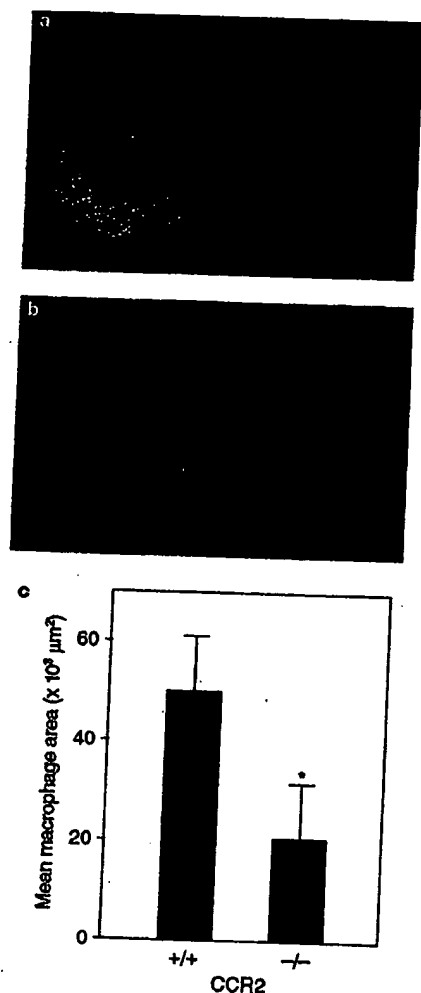


Figure 1 Macrophage infiltration of the aortic sinus in apoE^{-/-} mice fed a high-fat (Western) diet for 6 weeks. **a**, CCR2^{+/+}, **b**, CCR2^{-/-}. Sections from the aortic sinus were stained for macrophages with MOMA-2 (red) and for nuclei with SYTOX green. Regions of overlap appear yellow. Shown are representative sections from mice of each genotype. Original magnification $\times 125$. **c**, Quantitation of MOMA-2 staining. Values are means \pm s.d. (n = six mice of each genotype). * P = 0.0043 for CCR2^{+/+} versus CCR2^{-/-} (Mann-Whitney test).

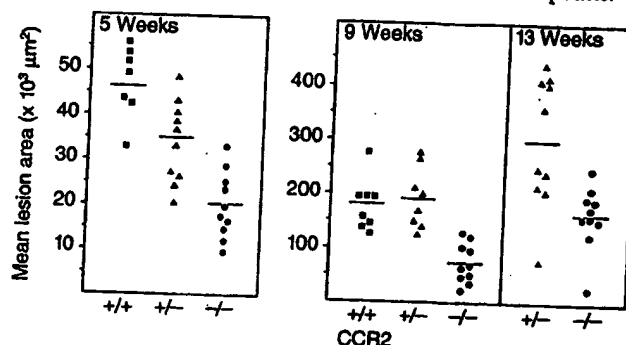


Figure 2 Mean lesion area in the aortic root. Cross-sections ($10\mu\text{m}$) of the aorta were stained for lipid with Oil Red O and quantitated by digital morphometry. Each symbol represents one animal; bars represent means. Spearman rank order analysis suggested a gene dosage effect at the 5-week time point ($r = 0.8029$, $P < 0.0001$). For comparisons between groups, $P < 0.001$ for CCR2^{+/+} versus CCR2^{-/-} and $P < 0.05$ for CCR2^{+/+} versus CCR2^{-/-} at 5 weeks (ANOVA); $P < 0.01$ for CCR2^{+/+} versus CCR2^{-/-} and $P < 0.001$ for CCR2^{+/+} versus CCR2^{-/-} at 9 weeks (ANOVA); $P = 0.0029$ for CCR2^{+/+} versus CCR2^{-/-} at 13 weeks (Mann-Whitney test).

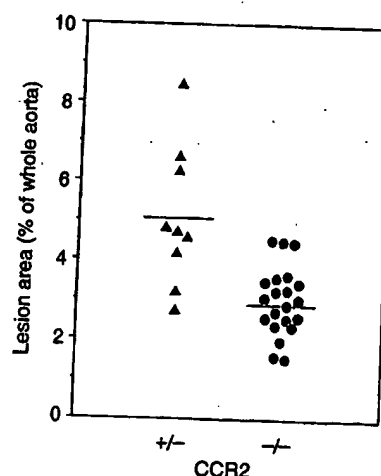


Figure 3 Lesion coverage in the aortas of CCR2^{+/+}, apoE^{-/-} and CCR2^{-/-}, apoE^{-/-} mice. Whole aortas from mice fed the high-fat diet for 13 weeks were mounted *en face* and stained for lipid with Sudan IV. Total lesion area from the aortic sinus to the femoral bifurcation was quantitated by digital morphometry. Each symbol represents one animal; bars represent means. The percentage of the aortic surface covered by lesions in the CCR2^{-/-} mice ($n = 21$) was significantly lower than in the CCR2^{+/+} mice ($n = 8$) ($P = 0.001$, Mann-Whitney test).

letters to nature

Table 1 Total cholesterol in serum from mice on the Western diet

Genotype	Total cholesterol (mg dl ⁻¹)		
	5 weeks	9 weeks	13 weeks
CCR2 ^{+/+} , apoE ^{-/-}	1816 ± 291 (17)	1947 ± 376 (8)	ND
CCR2 ^{+/+} , apoE ^{+/+}	1809 ± 408 (20)	1963 ± 354 (11)	1978 ± 402 (10)
CCR2 ^{-/-} , apoE ^{-/-}	1861 ± 431 (17)	1851 ± 513 (11)	1788 ± 376 (21)

Values shown are means ± s.d. (numbers of mice). ND, no data. Cholesterol levels were not significantly different between genotypes. $P = 0.1082$ for 5 weeks and $P = 0.8060$ for 9 weeks by ANOVA; $P = 0.1832$ for 13 weeks by Mann-Whitney test.

We next examined lesion development along the entire aorta in whole-mount *en face* preparations stained with Sudan IV. In animals on the Western diet for 5 and 9 weeks, less than 1% of the aorta was covered with lesions, and no significant differences between the CCR2 genotypes were observed (data not shown). At 13 weeks, however, lesions covered 5.1% of the aorta in the CCR2^{+/+} mice, but only 2.9% in the CCR2^{-/-} mice (Fig. 3). Thus, by two independent techniques, lesion size was significantly reduced in the absence of CCR2. Longer duration studies will be required to determine whether the size and complexity of the lesions in the CCR2^{-/-} mice eventually equal those of the wild-type animals.

To determine whether differences in lipid metabolism could account for the decreased atherosclerosis in the CCR2^{-/-} mice, we examined plasma lipid levels and lipoprotein profiles. Total serum cholesterol levels were not statistically different between the CCR2 genotypes at any time point and were within the range expected for apoE^{-/-} mice maintained on this diet¹¹ (Table 1). In addition, no differences were observed in plasma triglyceride levels (285.6 ± 107.7 mg dl⁻¹, $n = 6$ for CCR2^{+/+}; 282.8 ± 86.4 mg dl⁻¹, $n = 5$ for CCR2^{-/-}; 307.2 ± 79.6 mg dl⁻¹, $n = 7$ for CCR2^{+/+}). As analysed by gel filtration chromatography, the plasma lipoprotein profiles were essentially identical in wild-type and CCR2^{-/-} mice (Fig. 4). Consistent with earlier reports, most of the cholesterol in apoE^{-/-} mice was contained within very-low-density lipoprotein-sized particles⁸. Lesion size in apoE^{-/-} mice can be influenced by high-density lipoprotein (HDL) levels¹⁰, but HDL cholesterol levels were very low and were unaffected by the CCR2 genotype (Fig. 4). These data suggest that the decreased lesion size in the CCR2^{-/-} mice was not accounted for by changes in plasma cholesterol levels or in the distribution of lipoprotein particles. Gupta *et al.*¹³ reported decreased atherosclerosis in interferon- γ receptor-deficient mice on the apoE^{-/-} background and also noted an increase in plasma levels of apoA-IV, a potentially atheroprotective lipoprotein¹⁴. We found no difference in plasma apoA-IV concentrations between CCR2^{+/+} and CCR2^{-/-} mice (data not shown). The CCR2^{-/-} mice were backcrossed with C57Bl/6 mice, resulting in study animals that

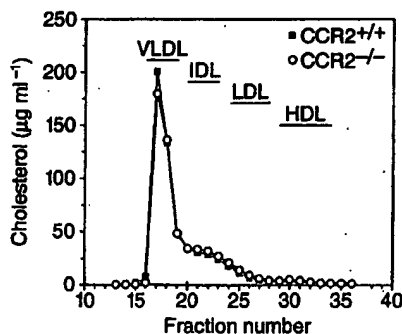


Figure 4 Lipoprotein profile of plasma from CCR2^{+/+} and CCR2^{-/-} mice. Values are averages of four mice of each genotype with similar total cholesterol levels. There were no significant differences in total non-HDL cholesterol (CCR2^{+/+}, $1,664 \pm 151$ mg dl⁻¹; CCR2^{-/-}, $1,510 \pm 210$ mg dl⁻¹) or HDL cholesterol (CCR2^{+/+}, 49 ± 27 mg dl⁻¹; CCR2^{-/-}, 54 ± 3.4 mg dl⁻¹) (Mann-Whitney). LDL, low-density lipoprotein; IDL, intermediate-density lipoprotein.

were 87.5% C57Bl/6 and 12.5% 129/Sv. Although unlikely, the possibility that a 129/Sv gene contributed to the differences in atherosclerosis cannot be completely excluded, particularly if this gene was linked to CCR2.

In summary, these studies identify CCR2 as a genetic determinant of murine atherosclerosis and provide strong evidence for a direct non-cholesterol-mediated effect of MCP-1 in macrophage recruitment and atherogenesis. These results are consistent with those of Suzuki *et al.*¹⁵, who reported a role for the macrophage scavenger receptor in atherosclerosis susceptibility. Monocytes also express CCR1 and CCR5, and atherosclerosis studies of mice in which multiple receptors have been genetically deleted will probably be of great interest. □

Methods

Genetically modified mice. CCR2^{-/-} mice⁷ (hybrids of C57Bl/6 and 129/SvJae) were mated with apoE^{-/-} mice⁹ on the C57Bl/6 background (N10 C57Bl/6, Jackson Labs). CCR2^{+/+}, apoE^{-/-} mice were backcrossed with apoE^{-/-} mice to produce CCR2^{+/+}, apoE^{-/-} mice; these mice were intercrossed to produce all three CCR2 genotype on the apoE^{-/-} background (87.5% C57Bl/6; 12.5% 129/SvJae). The double-knockout (apoE^{-/-}, CCR2^{-/-}) mice were born at the expected Mendelian ratios, developed normally, and were disease-free while maintained in a pathogen-free environment. Mice were weaned at 4 weeks, fed normal rodent chow (4.5% fat; Ralston Purina Co.) for an additional week, and switched to the Western diet (21% fat, 0.15% cholesterol; Harlan Tekland no. 88137) at 5 weeks of age¹¹.

Quantitation of atherosclerotic lesions. Hearts and aortas were prepared essentially as described^{16,17}, except that mice were perfused with 3.0% paraformaldehyde in PBS and tissues were equilibrated in 20% sucrose before embedding in OCT compound (Tissue Tek). Atherosclerosis was assessed by two independent techniques: Oil Red O staining of lesions in cross-sections from the aortic root and Sudan IV staining of lesions in pinned-out aortas. Cryosections (10 μ m) spanning 550 μ m of the proximal aorta were collected, and every fifth section, extending 250 μ m in both directions from the coronary artery branch point, was stained with Oil Red O. Lesion areas were quantified with a digital colour video camera and Image-1/AT software as described¹⁴. Adjacent sections were stained with haematoxylin and eosin for morphological analysis. The remainder of the aorta was opened longitudinally along the ventral midline from the aortic root to the iliac arteries, and the lesion area in *en face* preparations stained with Sudan IV was quantitated as described¹⁷. Image analysis was performed by a trained observer blinded to the genotype of the mice.

Immunohistochemistry. Serial cryosections collected at 50- μ m intervals were stained with a monoclonal rat antibody to the mouse monocyte-macrophage marker MOMA-2 (Biosource International, Camarillo, CA; 1:400 dilution), followed by detection with biotinylated secondary antibodies and streptavidin-horseradish peroxidase (PharMingen, San Diego). The signal was enhanced with the NEN DuPont Tyramide Signal Amplification kit, and sections were counterstained for nuclei with SYTOX green (Molecular Probes, Eugene, OR). Fluorescent images were captured into Image-1 as described above. Data represent the average area of MOMA-2 staining from 6–8 sections per mouse.

Lipid analysis. Cholesterol and triglyceride concentrations were determined with colorimetric assay kit (Spectrum cholesterol kit, Abbott Labs; GPO triglyceride kit, Boehringer Mannheim). Cholesterol levels were measured in serum of nonfasted mice collected at the time of killing, and triglycerides were assayed in plasma obtained after a 4-h fast. For lipoprotein analysis, plasma was prepared from blood collected by retro-orbital puncture into tubes containing EDTA (1 mM) and aprotinin (1.13×10^5 U ml⁻¹, ICN). Plasma samples from individual mice (50 μ l in a total volume of 300 μ l) were fractionated by FPLC on a Superpose 6 column (HR 10/30, Pharmacia LKB) as described¹⁶. ApoA-IV levels in plasma were determined by gradient gel electrophoresis (SDS-PAGE, 4–15% Tris-HCl Ready Gel; Bio-Rad) of very-low-density lipoprotein (fractions 17–19; Fig. 4) from four CCR2^{+/+} and four CCR2^{-/-} mice, followed by western blot analysis with a rabbit polyclonal antibody against rat apoA-IV (generously provided by Karl Weisgraber, Gladstone Institute of Cardiovascular Disease).

Statistical analysis. For analysis of lesion size, comparisons between groups were performed using the Kruskal–Wallis nonparametric ANOVA or Mann–

Whitney U test. The Spearman rank order correlation was used to analyse the relationship between CCR2 gene dosage and lesion area. Instat 2.01 software for the Macintosh was used for all calculations.

Received 11 May; accepted 3 July 1998.

- Cocchi, R. et al. Identification of RANTES, MIP-1 α , and MIP-1 β as the major HIV-suppressive factors produced by CD8⁺ T cells. *Science* 270, 1811–1815 (1995).
- Choe, H. et al. The β -chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* 85, 1135–1148 (1996).
- Nelken, N. A., Coughlin, S. R., Gordon, D. & Wilcox, J. N. Monocyte chemoattractant protein-1 in human atherosclerotic plaques. *J. Clin. Invest.* 88, 1121–1127 (1991).
- Yla-Herttuala, S. et al. Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc. Natl Acad. Sci. USA* 88, 5252–5256 (1991).
- Yu, X. et al. Elevated expression of monocyte chemoattractant protein 1 by vascular smooth muscle cells in hypercholesterolemic primates. *Proc. Natl Acad. Sci. USA* 89, 6953–6957 (1992).
- Cushing, S. D. et al. Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc. Natl Acad. Sci. USA* 87, 5194–5198 (1990).
- Boring, L. et al. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J. Clin. Invest.* 100, 2552–2561 (1997).
- Plump, A. S. et al. Severe hypercholesterolemia and atherosclerosis in apolipoprotein E-deficient mice created by homologous recombination in ES cells. *Cell* 71, 343–353 (1992).
- Zheng, S. H., Reddick, R. L., Piedrahita, J. A. & Maeda, N. Spontaneous hypercholesterolemia and arterial lesions in mice lacking apolipoprotein E. *Science* 258, 468–471 (1992).
- Plump, A. S., Scott, C. J. & Breslow, J. L. Human apolipoprotein A-I gene expression increases high density lipoprotein and suppresses atherosclerosis in the apolipoprotein E-deficient mouse. *Proc. Natl Acad. Sci. USA* 91, 9607–9611 (1994).
- Nakashima, Y., Plump, A. S., Raines, E. W., Breslow, J. L. & Ross, R. ApoE-deficient mice develop lesions of all phases of atherosclerosis throughout the arterial tree. *Arterioscler. Thromb.* 14, 133–140 (1994).
- Krasl, G., Rep, M. & Jensen, M. Macrophages in T and B cell compartments and other tissue macrophages recognized by monoclonal antibody MOMA-2. An immunohistochemical study. *Scand. J. Immunol.* 26, 653–661 (1987).
- Gupta, S. et al. IFN- γ potentiates atherosclerosis in apoE knock-out mice. *J. Clin. Invest.* 99, 2752–2761 (1997).
- DuVerger, N. et al. Protection against atherogenesis in mice mediated by human apolipoprotein A-IV. *Science* 273, 966–968 (1996).
- Suzuki, H. et al. A role for macrophage scavenger receptors in atherosclerosis and susceptibility to infection. *Nature* 386, 292–296 (1997).
- Purcell-Hayniah, D. A. et al. Transgenic mice expressing high levels of human apolipoprotein B develop severe atherosclerotic lesions in response to a high-fat diet. *J. Clin. Invest.* 95, 2246–2257 (1995).
- Venkant, M. M. et al. Susceptibility to atherosclerosis in mice expressing exclusively apolipoprotein B48 or apolipoprotein B100. *J. Clin. Invest.* 100, 180–188 (1997).

Acknowledgements. We thank D. Sanan, D. Newland and L. Jensen for help with tissue preparation and staining; D. Diczek for advice on statistical analysis; J. C. W. Carroll, N. C. Shew and S. Gonzales for figure preparation; J. Ernst for assistance with fluorescent microscopy; G. Howard and S. Ordway for editorial expertise; and A. Chen for manuscript preparation. This work was supported in part by a grant from the NIH to L.E.C.

Correspondence and requests for materials should be sent to L.E.C. at the Gladstone Institute of Cardiovascular Disease (e-mail: lcharo@gadstone.ucsf.edu).

Leptin modulates the T-cell immune response and reverses starvation-induced immunosuppression

Graham M. Lord^{††}, Giuseppe Matarese^{††}, Jane K. Howard[†], Richard J. Baker[†], Stephen R. Bloom[†] & Robert I. Lechler^{*}

Imperial College School of Medicine, Departments of *Immunology and †Endocrinology, The Hammersmith Hospital, Du Cane Road, London W12 0NN, UK

[†] These authors contributed equally to this work.

Nutritional deprivation suppresses immune function^{1–3}. The cloning of the *obese* gene and identification of its protein product leptin⁴ has provided fundamental insight into the hypothalamic regulation of body weight^{5,6}. Circulating levels of this adipocyte-derived hormone are proportional to fat mass^{6,7} but may be lowered rapidly by fasting^{8,9} or increased by inflammatory mediators^{10,11}. The impaired T-cell immunity of mice^{12,13} now known to be

defective in leptin (*ob/ob*)⁴ or its receptor (*db/db*)^{14,15}, has never been explained. Impaired cell-mediated immunity^{1–3} and reduced levels of leptin⁷ are both features of low body weight in humans. Indeed, malnutrition predisposes to death from infectious diseases¹⁶. We report here that leptin has a specific effect on T-lymphocyte responses, differentially regulating the proliferation of naive and memory T cells. Leptin increased Th1 and suppressed Th2 cytokine production. Administration of leptin to mice reversed the immunosuppressive effects of acute starvation. Our findings suggest a new role for leptin in linking nutritional status to cognate cellular immune function, and provide a molecular mechanism to account for the immune dysfunction observed in starvation.

Most immune responses are orchestrated by CD4⁺ helper T cells (Th). We first determined the effect of leptin on Th responses in the context of the mixed-lymphocyte reaction (MLR) resulting from the culture of T cells with major histocompatibility complex (MHC)-incompatible (allogeneic) stimulator cells. The doses of leptin used in these experiments were chosen to incorporate the range of serum levels measured in humans¹⁷. Leptin induced a

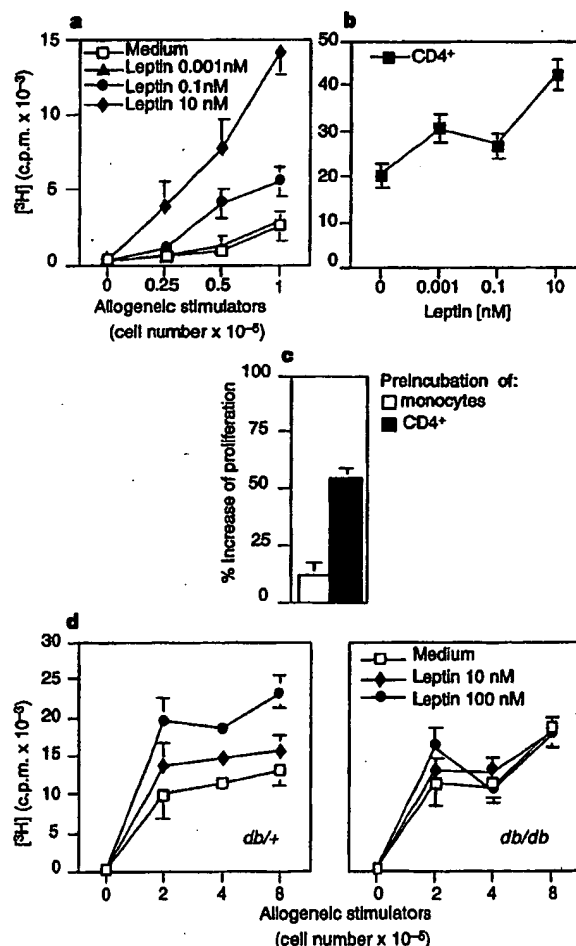


Figure 1 Leptin enhances the alloproliferative response. **a**, **b**, Thymidine incorporation in the presence of leptin in an MLR performed using human PBLs as responder and MHC-mismatched PBMCs as stimulator cells (**a**) or highly purified CD4⁺ T cells as responders as a responder/stimulator ratio of 1:1 (**b**) (6 experiments). **c**, Preincubation of either responder CD4⁺ T cells or irradiated stimulator allogeneic monocytes with 10 nM leptin before co-culture in the absence of leptin. **d**, MLR using murine heterozygous *db/+* (H-2^d) and homozygous *db/db* (H-2^b) splenocytes as responders with irradiated C57BL/6 (H-2^b) allogeneic splenocytes (3 experiments). All data are expressed as mean c.p.m. of triplicate cultures \pm s.e.m.

^{††} Present address: Laboratorio di Immunologia, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli "Federico II", via Pansini 5, 80131, Napoli, Italy.

*** TX REPORT ***

TRANSMISSION OK

TX/RX NO	1882
RECIPIENT ADDRESS	15712738300#1471
DESTINATION ID	
ST. TIME	03/17 15:33
TIME USE	28'12
PAGES SENT	34
RESULT	OK

Atty Docket No. 02307K-085041US

PTO FAX NO.: 1-571-273-8300

ATTENTION: Examiner Kolker, Daniele

Group Art Unit 1649

OFFICIAL COMMUNICATION
FOR THE PERSONAL ATTENTION OF
EXAMINER Kolker, Daniele

CERTIFICATION OF FACSIMILE TRANSMISSION

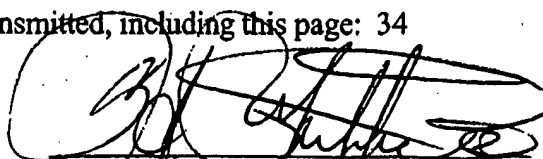
I hereby certify that the following documents in re Application of Charo et al., Application No. 10/791,166, filed March 1, 2004 for MAMMALIAN MONOCYTE CHEMOATTRACTANT PROTEIN RECEPTORS are being facsimile transmitted to the Patent and Trademark Office on the date shown below.

Documents Attached

1. Amendment w/3 references
2. Petition for Extension of Time Under 37 CFR 1.136(a)

Number of pages being transmitted, including this page: 34

Dated: March 17, 2006


Stanbanis I. Whitehurst

POTENTIAL ROLE OF MONOCYTE CHEMOATTRACTANT PROTEIN 1/JE IN MONOCYTE/MACROPHAGE-DEPENDENT IgA IMMUNE COMPLEX ALVEOLITIS IN THE RAT¹

MICHAEL L. JONES, MICHAEL S. MULLIGAN, CRAIG M. FLORY,
PETER A. WARD, AND JEFFREY S. WARREN²

From the Department of Pathology, University of Michigan Medical School, Ann Arbor, MI 48109-0602

We have examined the role of monocyte chemoattractant protein 1 (MCP 1) in the pathogenesis of monocyte/macrophage-dependent IgA immune complex alveolitis in the rat. Rat MCP 1 was cloned and expressed in order to facilitate analysis of its function in rat models of human disease. A cDNA library was constructed from rat pulmonary artery endothelial cells stimulated with TNF- α . The cDNA library was screened with synthetic oligonucleotide probes based on the recently published rat MCP 1 cDNA sequence. Among numerous MCP 1-positive clones, four full length (approximately 480 bp) cDNA were rescued, amplified by polymerase chain reaction, and ligated into a pJVTZ baculovirus transfer vector. *Spodoptera frugiperda* insect cells (Sf-21) infected with baculovirus recombinants (*Autographa californica* nuclear polyhedrosis virus) bearing properly oriented MCP 1 cDNA (AcMCP 1) directed the expression of unique peptides of 18, 21, and 23 kDa. Treatment of AcMCP 1-infected Sf-21 cells with tunicamycin resulted in reduced production of the 21- and 23-kDa proteins and an increase in 16- to 18-kDa products, the predicted size range of uncleaved and nonglycosylated rat MCP 1. Denatured and refolded 23-kDa and 21-kDa rat MCP 1 species exhibited dose-dependent monocyte-specific chemotactic activity at concentrations as low as 10^{-10} M whereas the 18-kDa species exhibited negligible activity. Antibodies that react with the 18-kDa, 21-kDa, and 23-kDa MCP 1 bands by Western immunoblot, block rat rMCP 1-directed monocyte chemotaxis, and neutralize monocyte-specific chemotactic activity secreted by TNF-stimulated rat endothelial cells were raised in rabbits immunized with the 23-kDa MCP 1 species. Intravenous administration of anti-MCP 1 antibodies upon initiation of IgA immune complex lung injury resulted in a marked reduction in lung injury as measured by pulmonary vascular permeability, alveolar hemorrhage, and pulmonary monocyte/macrophage recruitment. These data suggest that MCP 1 may play an important role in the pathogenesis of monocyte/

macrophage-dependent IgA immune complex alveolitis in the rat.

Despite the putative importance of IgA in diseases such as IgA nephropathy, Henoch-Schönlein purpura, dermatitis herpetiformis, and some cases of SLE, little is known about the pathogenesis of IgA-triggered tissue injury. We previously described an IgA immune complex-mediated lung injury model in the rat (1, 2). In contrast to various models of IgG immune complex-mediated tissue injury, IgA-induced injury develops fully in neutrophil-depleted rats (2). IgA immune complex lung injury requires an intact C system and is oxygen radical mediated (1, 2). Ultrastructural cytochemical analysis suggests that local production of H₂O₂ by mononuclear phagocytes is an important effector mechanism in IgA lung injury (3). In IgA lung injury monocytes and macrophages are recruited into the parenchyma and can be retrieved in BAL³ fluid during the development of injury. In contrast to IgG immune complex lung injury, in which locally produced TNF mediates neutrophil recruitment, negligible TNF activity can be detected in the BAL fluid of rats with IgA lung injury (4). The mechanisms through which monocytes and macrophages are recruited into the lungs of rats with evolving IgA immune complex-triggered alveolitis are unknown. A potential mediator of monocyte and macrophage recruitment is the monocyte chemoattractant, MCP 1.

MCP 1, known also as monocyte chemotactic and activating factor, is now known to be identical to the murine JE gene product (5). JE was described in 1983 as a gene that is expressed in mouse 3T3 fibroblasts after stimulation with platelet-derived growth factor (6). Although the JE gene product was not identified until later, its cellular sources and the stimuli promoting its expression were studied extensively (reviewed in Reference 7). MCP 1 is secreted either constitutively, or after induction with mitogens, cytokines, or growth factors, in a variety of cell types including lymphocytes, fibroblasts, endothelium, smooth muscle, and several tumor cell lines (reviewed in Reference 7). In vitro studies indicate that MCP 1 can enhance the tumorigenic activity of monocytes against several lines of tumor cells and that it is a potent chemotactic factor for monocytes (7-10).

Although in vitro studies have provided invaluable in-

Received for publication March 31, 1992.

Accepted for publication June 30, 1992.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ This work was supported, in part, by the National Institutes of Health (HL-40526, HL-48287 and 5T32-HL-07517).

² Address correspondence and reprint requests to Jeffrey S. Warren, M.D., Department of Pathology, The University of Michigan Medical School, 1301 Catherine Street, Box 0602, Ann Arbor, MI 48109-0602.

³ Abbreviations used in this paper: BAL, bronchoalveolar lavage; MCP 1, monocyte chemoattractant protein 1; PCR, polymerase chain reaction; Sf-21, *Spodoptera frugiperda*-21; AcNPV, *Autographa californica* nuclear polyhedrosis virus; AcMCP 1, *Autographa californica* MCP 1; T-TBS, Tween-Tris-buffered saline; mol, multiplicity of infection; DNP-BSA, dinitrophenol-conjugated BSA.

sights into the potential functions of MCP 1, little is known about its role in physiologic or pathologic processes. The *in vitro* activities ascribed to MCP 1 suggest that it may be of fundamental importance as an inducer of the monocyte/macrophage-rich lesions that are characteristic of such pathologic processes as atherosclerosis, chronic inflammation, infiltration of tumors by monocytes, and granulomatous inflammation. Indeed, recent immunohistochemical and *in situ* hybridization analyses have revealed MCP 1 expression within macrophage-rich foci of atherosclerotic lesions in human and rabbit arteries (11, 12). Northern hybridization analyses of whole rat lungs containing glucan-induced granulomas indicate that MCP 1 gene expression is up-regulated during granuloma formation (13). Analyses of the biologic function of MCP 1 in such processes have not yet been carried out. In order to study the functional role of this mediator in rat models of human disease we cloned and expressed rat MCP 1.

In this study, we addressed the pathophysiologic role of MCP 1 in IgA immune complex-induced lung injury in the rat. This is an ideal model because lung injury is mediated by monocytes and macrophages and can be quantitated using several parameters. The data suggest that MCP 1 may play an important role in the pathogenesis of IgA immune complex alveolitis in the rat.

MATERIALS AND METHODS

Materials. *Spodoptera frugiperda* insect cells (Sf-21; B821-01), wild-type baculovirus stock (*Autographa californica* nuclear polyhedrosis virus; AcNPV), β -galactosidase recombinant stock (Ac-pJVETLZ), and AcNPV wild-type DNA were purchased from Invitrogen, San Diego, CA. The pJVETLZ transfer vector, (pBlueBac; a modification of pJVP10) (14), which contains the polyhedrin gene promoter, an *NheI* restriction site, an early transcriptase locus early promoter, and a β -galactosidase-coding region, was kindly provided by Dr. Chris Richardson (Biotechnical Research Institute, Montreal, Canada). Grace's insect cell culture medium was from GIBCO Laboratories, Grand Island, NY. FCS was from GIBCO. Streptomycin, penicillin, and fungizone were from Whittaker Bioproducts, Walkersville, MD. Sea plaque low melting agarose was from FMC Bioproducts, Rockland, ME. Restriction enzymes and other molecular biology materials were obtained from Boehringer-Mannheim Biochemicals, Indianapolis, IN; Bethesda Research Laboratories, Gaithersburg, MD; and New England Biolabs, Boston, MA. Synthetic oligodeoxynucleotides were prepared by the DNA Synthesis Facility (D. L. Oxender, Ph.D., Director), University of Michigan, Ann Arbor, MI.

Rat pulmonary artery endothelial cells. Rat pulmonary artery endothelial cells stimulated with human TNF- α were used for con-

Construction and screening of rat cDNA library. Total RNA was isolated by the guanidinium-isothiocyanate method (16) from rat pulmonary artery endothelial cells stimulated with human rTNF- α (200 U/ml) for 4 h. Poly (A) RNA was isolated by oligo(dt)-cellulose chromatography (16). cDNA synthesized by a modification of the Gubler and Hoffman method (17) was used to construct a library in pCDNA II (Invitrogen, San Diego, CA). pCDNA II (bearing an ampicillin-resistance gene) was electroporated into DH1aF' *Escherichia coli* cells and the library amplified on LB + ampicillin (50 μ g/ml) plates. A [³²P]PCR-labeled oligodeoxynucleotide probe (5'-TAC AGC TTC TTT GGG ACA CCT GCT GGT GAT-3'), complementary to positions 160-193 of the rat MCP 1 cDNA sequence provided by T. Yoshimura et al. (18), was used to screen the cDNA library by high density plaque hybridization (16). Hybridization to nitrocellulose filters was carried out at 37°C in 6X SSC, 5X Denhardt's solution, 0.05% sodium pyrophosphate, 1% SDS, 100 μ g/ml salmon sperm DNA, and 10⁶ dpm/ml probe. Filters were washed three times for 5 min with 6X SSC and 0.1% SDS at 37°C, three times for 30 min at 35°C, dried, and exposed to XAR-5 film (Kodak, Rochester, NY) at -70°C with intensifying screens. Appropriately sized MCP 1 cDNA were selected by successful PCR (Taq polymerase; Cetus Immune) amplification of cDNA using primer pairs that contained *NheI* restriction sites and bracketed the 5' and 3' termini of the rat MCP 1 cDNA sequence published by Yoshimura et al. (18). The primer sequences were: 5'-ATC^{NheI} AGC TAG CCT CCA CCA CTA TGC-3' and 3'-CTA AAC CTT ACA CTA^{NheI} CGA TCG GGT GG-5'.

Construction of the baculovirus transfer vector. The resulting MCP 1 cDNA PCR product of one of the selected full length rat MCP 1 clones (clone 2B) was cut with *NheI* according to manufacturer's instructions (New England Biolabs) and the DNA separated in 1% agarose (16). The resulting band was then cut out of the gel and purified using the following two-microfuge tube method. A hole was punched through the bottom of a 500- μ l tube containing angel hair and this tube (to which the DNA sample was added) was placed inside of a larger microfuge tube which was then centrifuged at 14,000 rpm for 5 min. The preparation was extracted in phenol/chloroform/indole acetic acid, the aqueous phase precipitated in cold ethanol, and the resulting DNA quantitated (16). The DNA pellet was then added to ligation mixes (with molar ratios of insert:vector of 0.5:1, 1:1, 2:1, and 4:1), which contained pJVETLZ that had been linearized with *NheI* and phosphatased (16). As a negative control pJVETLZ alone was subjected to the same set of conditions. The ligation products (and controls) were transfected into *E. coli* INV1aF' (DH1 derivative) (Invitrogen) and amplified. MCP 1-positive colonies (see plaque hybridization protocol above) were picked, grown overnight in fungizone ampicillin growth medium, and subjected to DNA extraction (miniprep method) (16). Preparations containing appropriately sized MCP 1 cDNA inserts (\approx 480 bp) were then analyzed for proper insert orientation.

Proper orientation of the rat MCP 1 insert within the pJVETLZ transfer vector was assessed by using the oligonucleotide primer pairs that were complementary to a portion of the insert containing rat MCP 1 (near the 3'-end) and a portion of the transfer vector at a site adjacent to the 5' end of the MCP 1 insert. Using this method only properly oriented rat MCP 1 inserts would be expected to yield an appropriately sized (\approx 480 bp) PCR product. In addition, if insert orientation direction was random, approximately one-half of the insert-bearing vectors would be expected to yield a product. The primers were: "Vector" primer for pJVETLZ;

5'-GCC GGA TTA TTC ATA CCG TC-3' and
[-34 -from *NheI* site - -15]

"Insert" primer for rat MCP 1: 3'-CTA AAC CTT ACA CTA CGA TCG GGT GG-5'.
[451 - 467]

struction of a cDNA library. The cells were extracted from rat pulmonary arteries by perfusion with microcarrier beads, characterized as endothelial, and maintained in culture as previously described by Ginsburg et al. (15). Cells (sixth passage) used for RNA extraction were grown to confluence in 150 cm² T-flasks (Corning Glass, Corning, NY). The day before RNA extraction the culture medium was removed, the monolayers washed twice with HBSS (GIBCO), and the medium replaced with RPMI 1640 (GIBCO) without serum, endothelial cell growth supplement, or heparin. Upon initiation of stimulation, endothelial monolayers were again washed and fresh medium without growth factors was added along with 200 U/ml rTNF- α (Cetus Immune, Emeryville, CA). After 4 h, culture medium was collected and RNA was prepared from the endothelium as described below. For molecular sizing of native rat MCP 1 serum-free conditioned medium was collected after 12 h.

Transfection of *S. frugiperda* cells and isolation of recombinant virus. The MCP 1-bearing transfer vector (AcMCP 1), amplified in *E. coli* INV1aF', was mixed with wild-type AcNPV DNA (2 μ g/1 μ g) and co-precipitated with calcium phosphate (16). This mixture was used to transfect monolayers of Sf-21 cells maintained at a density of 2.0 \times 10⁶ cells/25-mm tissue culture dish. After 4 h the transfection mix was removed and the monolayers washed twice with Grace's medium. After 4 days, serial dilutions (10⁻¹ to 10⁻⁶) of culture supernatant were layered onto 75% confluent monolayers of Sf-21 cells, incubated for 1 h, washed, and overlaid with fresh Grace's medium containing 1% agarose (Sea plaque) and Blue-O-gal substrate (150 μ g/ml; GIBCO). The cultures were maintained for 4 days at 27°C by which time many cells contained occlusion bodies characteristic of the polyhedrin protein. Plaques devoid of occlusion bodies and positive for β -galactosidase expression (blue in the presence of Blue-O-

gal) were purified and amplified by two successive rounds of plaque assays. *S. frugiperda* cells were grown and maintained at 27°C in Grace's insect medium (GIBCO) supplemented with 10% FCS, penicillin (50 U/ml), streptomycin (50 µg/ml), and fungizone (50 µg/ml) as described by Summers and Smith (19). For amplification of virus, wild-type baculovirus (AcNPV), or recombinant virus (AcMCP 1) was used to infect Sf-21 cells at a mol of 1.0 plaque-forming units/cell. Negative controls included wild-type virus (AcNPV), rMCP 1-negative virus (AcpJVETLZ), and noninfected Sf-21 cells.

Production of rat MCP 1 in Sf-21 cells. Recombinant virus stocks were grown from second passage virus in Sf-21 cells to obtain high titers ($>10^8$ plaque-forming units/ml). Pilot experiments were conducted to establish the optimal mol and the optimal time after infection to harvest the cells. For high level rat MCP 1 expression, suspension cultures of Sf-21 cells (10^6 cells/ml) were infected at mol = 1, and harvested 96 h after infection by centrifugation (700 × g). Pelleted cells were washed with Tris/Cl (50 mM; pH 7), 1 mM dithiothreitol and 250 mM sucrose, resuspended in 0.1 vol Tris/Cl (50 mM; pH 7), 1 mM dithiothreitol and 0.5 M NaCl, and then subjected to three freeze-thaw cycles in liquid nitrogen. Culture supernatants, cell pellet lysates (after DNA was sheared by three passes through a 25-gauge needle), and resuspended (0.1 vol; 50 mM Tris/Cl, pH 7, 1 mM dithiothreitol) cell pellets were used in subsequent analyses and preparations.

Characterization of AcMCP 1 expression products. Proteins expressed by AcMCP 1 and negative controls (uninfected Sf-21 cells, Sf-21 cells infected with wild-type baculovirus (AcNPV), and Sf-21 cells transfected with expression vector alone (AcpJVETLZ)) were characterized by SDS/PAGE under reducing conditions on 13.5% gels stained with Coomassie blue R or silver (20, 21). Analysis of MCP 1 glycosylation was carried out by SDS/PAGE using Sf-21 cells infected with AcMCP 1 and incubated in the presence or absence of tunicamycin (10 µg/ml). In vitro biologic activity (monocyte and neutrophil chemotaxis) was assessed as described below.

Chemotaxis assays. Monocyte and neutrophil chemotaxis assays were carried out in parallel in 48-well microchemotaxis chambers as described by Falk et al. (22). Cell suspensions (2.25×10^5 cells/well) were added to the top well of the chamber and permitted to migrate through 10 µm polycarbonate membranes (5 µm porosity for monocytes and 3 µm porosity for neutrophils) toward sample-bearing bottom chambers. After a 4-h incubation (37°C, 5% CO₂, humidified) membranes were removed and the nonmigrating cells wiped off. The membranes were then fixed for 10 min in absolute methanol, air dried, and stained for 30 min in 2% toluidine blue. The numbers of cells migrating through the membrane were counted in three random, 10-mm grids at 400×, with the mean ± SEM calculated for triplicate samples. Results are expressed as normalized values representing the percent of maximum FMLP (10^{-8} or 10^{-9} M; as indicated) positive control, minus negative controls (buffer alone). Peripheral blood human monocytes (80% to 85%) were isolated by centrifugation through Ficoll-Hypaque (Sigma, St. Louis, MO) and Sepacell-MN (Sepratech, Oklahoma City, OK) as described by Vissers et al. (23). Peripheral blood human neutrophils (91% to 96%) were isolated by centrifugation through Ficoll-hypaque as described by Boyum (24).

Protein solubilization and refolding. Freeze-thaw lysates from AcMCP 1-infected Sf-21 cells were subjected to preparative SDS-PAGE (12.5%). Unfixed, unstained 18-kDa, 21-kDa, and 23-kDa MCP 1 bands, and where indicated, a 44-kDa baculoviral protein band, were cut out and electroeluted into 8 M urea plus 10 mM 2-ME. This was dialyzed against 2000 volumes of (1 mM) 2-ME in HBSS (48 h, 4°C).

Rabbit polyclonal anti-MCP 1. Polyclonal rabbit anti-rat MCP 1 was raised against the 23-kDa MCP 1 in 3 kg New Zealand White rabbits (Charles River Laboratories, Wilmington, MA) immunized with MCP 1 (50 µg) emulsified in Hunter's TiterMax (CytRx, Norcross, GA) and boosted after 1 month with MCP 1 (25 µg). Where indicated, the resulting antiserum was affinity purified using a protein A Sepharose column (Sigma). Anti-MCP 1 serum was diluted 1/1 with PBS (100 mM phosphate, pH 8.0, and 150 mM NaCl) and applied slowly to the PBS-washed column. After extensive washing with PBS, the column was stripped with 100 mM sodium acetate buffer, pH 3.0. One milliliter fractions were collected in tubes containing 50 µl of 1 M Tris buffer, pH 8.0. The affinity-purified IgG fraction was then dialyzed against PBS.

Western immunoblot. Sf-21 cell pellet lysates were subjected to SDS/PAGE (12.5%) according to the method of Laemmli (20). The separated proteins were transblotted to nitrocellulose (0.45 µm; Bio-Rad, Richmond, CA) for 1 h at 12 V with a Genie transblot apparatus (Ideal Scientific, Corvallis, OR). After transfer, the membrane was blocked with T-TBS (20 mM Tris-HCl, pH 7.5; 500 mM NaCl; 0.05% Tween 20) (v/v) containing 3% BSA, Cohn fraction V, (Sigma) for 2 h at room temperature. After removal of the blocking solution, the

blot was washed with T-TBS (5 min; three times). Primary antibody (affinity-purified rabbit anti-rat MCP 1; 1 mg/ml) was then added at final concentration of 50 µg/ml in T-TBS with 1% BSA (v/v) and incubated for 1 h. The primary antibody was decanted and the membrane washed as described above. After the final wash, secondary antibody (goat anti-rabbit IgG alkaline phosphatase-conjugated antibody (Bio-Rad) was added at a final dilution of 1/3000 in T-TBS with 1% BSA, and incubated for 1 h. The membrane was washed as above and the bands developed by addition of alkaline phosphatase substrate solution (5-bromo-4-chloro-3-indoyl phosphate and nitro-blue tetrazolium in 10 mM Tris; pH 9.5). Rainbow m.w. markers (Amersham, Arlington Heights, IL) were used to estimate m.w.

IgA antibodies and DNP/BSA Ag. Affinity-purified monoclonal IgA (MOPC 315) directed against DNP-BSA was purchased from Sigma. Dinitrophenol-conjugated BSA was prepared according to the method of Eisen (25). The resulting Ag preparation contained an average of 50 DNP groups/BSA molecule.

Rat IgA immune complex-induced alveolitis. Male Long-Evans pathogen-free rats (350 g; Charles River) were used for all studies. Intraperitoneal injections of ketamine (2.5 to 5.0 mg/100 g body weight) and sodium pentobarbital (5 mg/100 g body weight) were given for sedation and anesthesia. IgA immune complex lung injury was induced as previously described (1-4). Antibody solution (IgA anti-DNP/BSA; 400 µg) was instilled into the lungs through a tracheal cannula. In all cases, a final volume of 300 µl was instilled into the lungs. Ag (DNP-BSA; 3.3 mg) was injected i.v. Rats were killed at the indicated times, lung injury was quantitated, and BAL fluid was harvested. Anesthetized rats were exsanguinated through inferior vena caval section before removal of lungs, thus resulting in negligible contamination of lungs with blood. Pulmonary injury was quantitated by permeability, hemorrhage, and morphometric measurements. Permeability indices were calculated by comparing the leakage of ¹²⁵I-labeled bovine γ-globulin from the circulation into the lung to the ¹²⁵I-labeled colloid remaining in 1 ml of blood as previously described (1-4). Hemorrhage indices were calculated by comparing the leakage of ⁵¹Cr-labeled RBC from the circulation into the lung to the ⁵¹Cr-labeled RBC remaining in 1 ml of blood as previously described (1-4). Intravenous anti-MCP 1 or preimmune serum was infused at time zero.

Morphometric analysis of mononuclear phagocyte recruitment and alveolar hemorrhage. Lung samples (1 mm³) were excised from the peripheral aspect of whole lungs that had been fixed in 4% glutaraldehyde under constant pressure inflation (25 cm H₂O) (4). Samples were washed in 0.1 M cacodylate buffer (pH 7.3), embedded in 1 µm thick plastic sections, and stained with toluidine blue. Use of 1-mm³ samples from the peripheral aspects of inflated lung results in sections that contain no large bronchial structures. (Large bronchial structures are defined as muscular airways lined by respiratory epithelium). Plastic embedding (1 µm thick) allows very high morphologic resolution, thus allowing virtually all alveolar and alveolar septal cells to be easily identified. Morphometric analysis of mononuclear phagocyte recruitment and alveolar RBC (hemorrhage) was carried out by a pathologist (J. S. Warren) who was blinded to sample origin. For each condition, five samples were examined. In each sample, 45 to 60 randomly selected 40× microscopic fields (high power field) were analyzed.

Analysis of BAL fluid. Lung lavage contents for cell counts were collected using 5 ml of 37°C, serum-free RPMI 1640 (3, 4). At least 90% of the administered fluid was always recovered, centrifuged (400 × g; 7 min) to separate cells, and the cells were counted. There was no systematic difference in BAL fluid recovery between control and experimental groups of rats.

RESULTS

Rat MCP 1 cDNA cloning. Several studies have revealed that stimulation of human umbilical vein endothelial cells with TNF-α results in the expression of MCP 1 and the secretion of a monocyte-specific chemotactic protein (26-28). Accordingly, we constructed a cDNA library from rat pulmonary artery endothelial cells stimulated with human TNF-α and cloned a full length rat MCP 1 cDNA. The cDNA library, which contained greater than 1.2×10^6 ampicillin-resistant recombinants, was initially screened with an oligodeoxynucleotide probe that was complementary to positions 160-193 of the rat MCP cDNA sequence published by Yoshimura et al. (18). Among numerous MCP 1-positive colonies, four yielded products after PCR in which primers that bracketed the

5' and 3' termini of the published rat MCP 1 cDNA open reading frame were employed (18). Plasmid DNA preparations prepared from these four clones each yielded fragments of approximately 500 to 700 bp after digestion with *Bam*HI and *Spe*I (polylinker sites in the pCDNA II cloning vector). One cDNA clone (clone 2B) was then amplified by PCR using primer pairs that contained *Nhe*I restriction sites and bracketed the 5' and 3' termini of the rat MCP 1 cDNA sequence.

Expression of rat MCP 1 in a baculovirus system. The baculovirus AcNPV is a helper-independent expression vector that has been used successfully to express several eukaryotic genes (reviewed in Reference 29). A rat MCP 1 expression vector was prepared by ligating the full length MCP 1 cDNA clone 2B (at varying insert:vector ratios) into pJVETLZ. Although successful ligations occurred at each insert:vector ratio employed (0.5:1, 1:1, 2:1, 4:1), a 1:1 ratio was used for this construction because it yielded the maximum number of products bearing single insert copies (data not shown). Proper orientation of the rat MCP 1 insert within the pJVETLZ transfer vector was assessed using the PCR with oligonucleotide primer pairs that were complementary to a portion of the insert containing rat MCP 1 (near the 3' end) and a portion of the transfer vector at a site adjacent to the 5' end of the MCP 1 insert. Of seven MCP 1-bearing transfer vectors, four yielded PCR products that indicated proper insert orientation (Fig. 1). Using this method only properly oriented MCP 1 inserts would be expected to yield an appropriately sized (481-bp) PCR product. In addition, if the insert orientation is random, approximately one-half of the insert-bearing vectors would be expected to yield a product.

Recombinant virus (AcMCP 1) was obtained by homologous in vivo recombination between wild-type virus (AcNPV) and the MCP 1-bearing transfer vector. Recombinant viruses were purified by two serial passages as described in *Methods*. Sf-21 cells were infected with rAcMCP 1 (third passage), recombinant virus lacking the MCP 1 insert (AcpJVETLZ), or wild-type virus (AcNPV). Uninfected Sf-21 cells were also included as an additional negative control. AcMCP 1 directed the expression of 18-kDa, 21-kDa, and 23-kDa bands that were not present in uninfected Sf-21 cells or in Sf-21 cells infected with either AcNPV or AcpJVETLZ (Fig. 2). The yield of rat

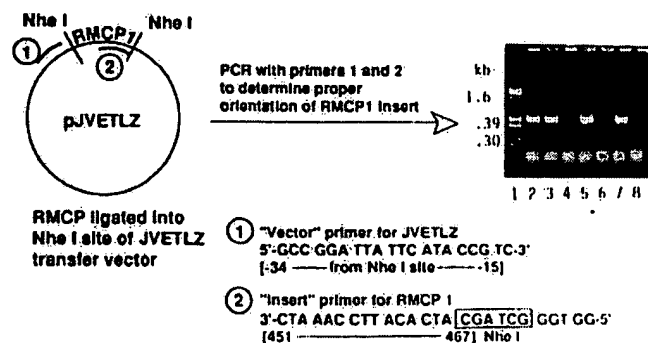


Figure 1. Proper orientation of rat MCP 1 (RMCP 1) in the pJVETLZ transfer vector. Lanes 2, 3, 5, and 7 contain rat MCP 1 insert ligated into pJVETLZ in the proper 5' → 3' orientation. The four properly oriented PCR products (lanes 2, 3, 5, and 7) migrate above the 0.39-kb size marker, at a location consistent with the expected 481-bp PCR product. PCR products were electrophoresed through 0.9% agarose and stained with ethidium bromide.

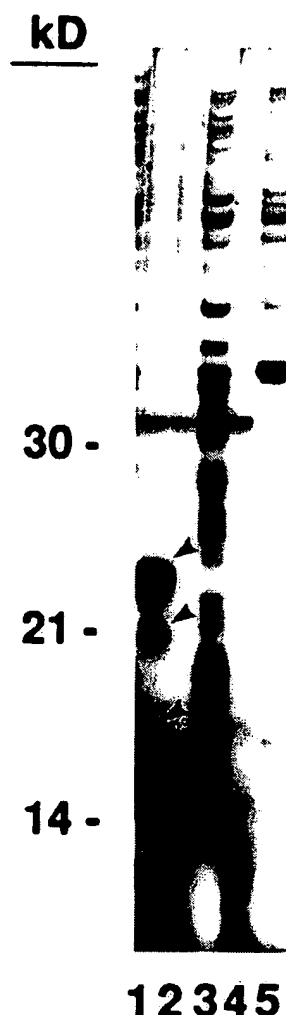


Figure 2. Expression of rat MCP 1 in Sf-21 cells. Sf-21 cells, grown at a density of 2.0×10^6 /25-mm culture dish, were infected with AcMCP 1 (lane 1), wild-type virus (AcNPV) (lanes 2 and 4), no virus (untreated Sf-21 cells) (lane 3), and recombinant virus lacking an MCP 1 insert (AcpJVETLZ) (lane 5). Protein bands of 18 kDa, 21 kDa, and 23 kDa (arrows) are present only in Sf-21 cells infected with AcMCP 1. These cells were harvested 96 h after infection and lysed in 100 μ l 50-mM Tris/Cl, pH 7, and 1 mM dithiothreitol. After addition of SDS sample buffer, fractions were boiled for 5 min and 20 μ l of each applied to a 13.5% polyacrylamide gel (20). After electrophoresis, the gel was stained with silver according to the method of Wray et al. (21). Lane kd indicates m.w. markers in kilodaltons.

MCP 1 per 2×10^6 starting Sf-21 cells increased as a function of time over 96 h (data not shown). Over the same time period, Sf-21 cells infected with AcpJVETLZ expressed increasing concentrations of β -galactosidase, but no protein in the 18- to 23-kDa range. No new protein bands were observed in Sf-21 cells infected with AcNPV or in uninfected Sf-21 cells at any of the time points examined (data not shown). Essentially all of the stainable rat MCP 1 (18 to 23 kDa protein) was recovered from solubilized Sf-21 cell lysates. The bulk of rat MCP 1 expressed by Sf-21 cells had a m.w. of 23 kDa. These data indicate that Sf-21 cells infected with AcMCP 1 direct the expression of proteins of 18 kDa, 21 kDa, and 23 kDa in a time-dependent manner over 96 h.

Characterization of rat rMCP 1. Based on an open reading frame of 444 bp (18), the predicted m.w. of unprocessed rat MCP 1 is 16.3 to 18.9 kDa. We employed tunicamycin to determine whether the 21-kDa and 23-

kDa protein bands might represent glycosylated species of rat MCP 1. SDS/PAGE analysis of solubilized lysate pellets from Sf-21 cells incubated with tunicamycin revealed several major bands between 16 and 18 kDa and small residual 21-kDa and 23-kDa bands (Fig. 3). This observation suggests that Sf-21 cells infected with AcMCP 1 express variably glycosylated protein species.

Solubilized lysates from Sf-21 cells infected with AcMCP 1 exhibited negligible monocyte or neutrophil chemotactic activity *in vitro* (data not shown). Accordingly, we sought to solubilize and renature the protein of interest in the hope of producing biologically active rat MCP 1. Cell lysates from AcMCP 1-infected Sf-21 cells (96-h cultures) were subjected to SDS-PAGE. Individual bands (18-kDa, 21-kDa, 23 kDa, and irrelevant 44-kDa baculoviral protein) were cut out, electroeluted, and solubilized in 8 M urea plus 10 mM 2-ME followed by dialysis for 48 h against 2000 volumes of HBSS (4°C) containing 1 mM 2-ME. Renatured cell pellet lysates from AcMCP 1-

infected Sf-21 cells exhibited no neutrophil chemotactic activity (data not shown). The 23-kDa and 21-kDa rat MCP 1 species exhibited dose-dependent, monocyte-specific chemotactic activity, whereas the 18-kDa MCP 1 species exhibited little activity (Fig. 4). As shown in Figure 4, the irrelevant 44-kDa baculoviral protein exhibited no monocyte chemotactic activity.

Characterization of anti-rat MCP 1. Western immunoblot analysis of anti-MCP 1 revealed specific reactivity with the 18-kDa, 21-kDa, and 23-kDa rat MCP 1 species (Fig. 5). Anti-rat MCP 1 serum specifically blocked rat rMCP 1 (23 kDa)-induced monocyte chemotaxis in a dose-dependent manner with 95% blockade of MCP 1 (10^{-9} M) activity with undiluted anti-rat MCP 1 serum (Fig. 6). Equivalent concentrations of preimmune serum exhibited less than 10% blockade of MCP 1-mediated monocyte chemotaxis.

The capacity for anti-rat MCP 1 to neutralize native monocyte chemotactic activity was tested using TNF- α -

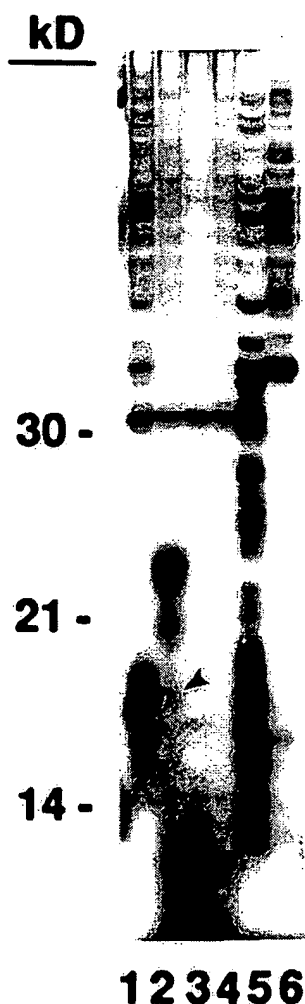


Figure 3. Effect of tunicamycin on rat MCP 1 expression. Addition of tunicamycin to Sf-21 cells infected with AcMCP 1 results in the production of a cluster of protein bands of approximately 16 to 18 kDa. Sf-21 cells, grown at a density of 2.0×10^6 /25-mm culture dish, were infected with AcMCP 1 in the presence of tunicamycin ($10 \mu\text{g/ml}$) (lane 1), AcMCP 1 in the absence of tunicamycin (lane 2), wild-type virus (AcNPV) (lanes 3 and 4), no virus (uninfected Sf-21 cells) (lane 5), and recombinant virus lacking an MCP 1 insert (AcJPVETLZ) (lane 6). These cells were harvested at 96 h after infection and processed as described in the legend for Figure 2. As in Figure 2, there is a small amount of 18-kDa protein (arrow) produced by Sf-21 cells infected with AcMCP 1 (lane 2).

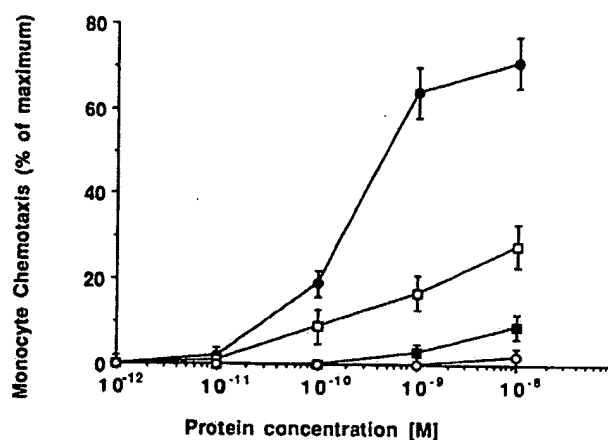


Figure 4. Renatured rat MCP 1 exhibits *in vitro* monocyte-specific chemotactic activity. The 23-kDa (—●—) and 21-kDa (—□—) MCP 1 species possess potent, dose-dependent monocyte chemotactic activity. The 18-kDa MCP 1 species (—■—) and an irrelevant 44-kDa viral protein (—○—) exhibit little and no monocyte chemotactic activity, respectively. The data (means \pm SEM) are expressed as percentage of maximum monocyte chemotactic response to FMLP (10^{-8} M). The average percentage of input monocytes that migrated in response to FMLP (10^{-8} M) was $27 \pm 4\%$. All samples were assayed in triplicate.

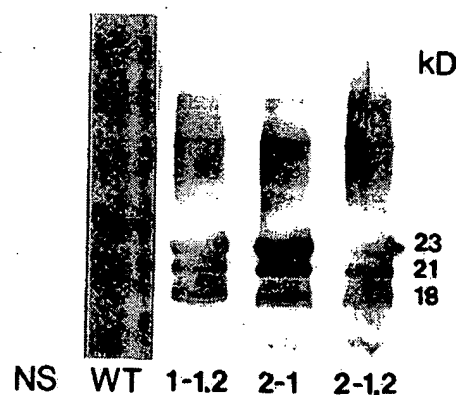


Figure 5. Rabbit polyclonal antibody raised against the 23-kDa rat MCP 1 reacts with the 18-kDa, 21-kDa, and 23-kDa rMCP 1 species. Western immunoblot was carried out as described in *Materials and Methods*. Preimmune serum from rabbit 2 (NS) did not react. Lane WT (wild type) represents reaction between anti-MCP 1 antiserum taken from rabbit 2 and reacted against cell pellet lysates from Sf-21 cells infected with AcNPV. Lanes 1-1.2, 2-1, and 2-1.2 represent anti-MCP 1 antisera obtained from two different rabbits. Antisera were heat inactivated (56°C , 30 min) before application.

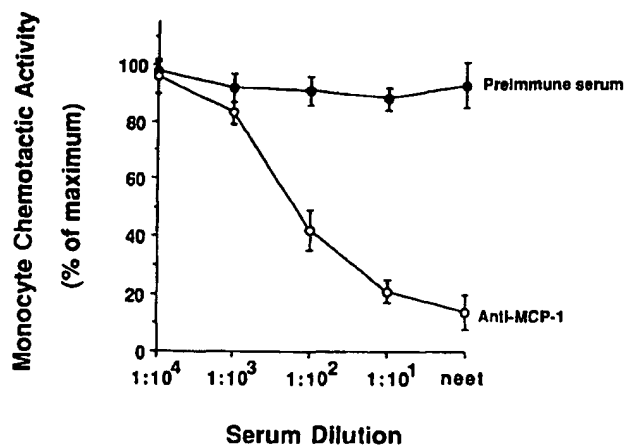


Figure 6. Neutralization of rMCP 1 monocyte chemotactic activity with anti-MCP 1 serum. Data (means \pm SEM; triplicate samples) are expressed as varying dilutions of antiserum vs monocyte chemotactic activity expressed as percentage of maximum response, where 100% is the response to 10^{-6} M MCP 1 (23 kDa). Sample chambers contained the indicated dilutions of heat-inactivated preimmune serum (\bullet) or anti-MCP 1 serum (\circ).

induced monocyte chemotactic activity secreted by rat endothelial cells. Rat pulmonary artery endothelial cells stimulated with human TNF- α expressed increased MCP 1 mRNA levels (data not shown) and secreted an 8- to 30-kDa monocyte-specific chemotactic activity (data not shown), which could be blocked in a dose-dependent manner with anti-rat MCP 1 (62% reduction in monocyte chemotaxis with a 1/100 dilution of anti-MCP 1 serum).

These data indicate that the anti-rat MCP 1 raised against 23-kDa rMCP 1 cross-reacts with the 18- and 21-kDa rMCP 1 species and that it specifically blocks rat rMCP 1 functional activity in vitro. Blockade of the 8- to 30-kDa monocyte-specific chemotactic activity secreted by TNF- α -stimulated rat pulmonary artery endothelial cells indicates that anti-MCP 1 neutralizes native rat MCP 1 activity. It should be noted that anti-MCP 1 had no blocking effect on FMLP (10^{-6} M)-mediated monocyte chemotaxis, zymosan-activated serum-mediated monocyte chemotaxis, or neutrophil chemotaxis (FMLP, 10^{-9} M; zymosan-activated serum) (data not shown).

Role of MCP 1 in pathogenesis of IgA immune complex-triggered alveolitis. Intravenous infusion of anti-MCP 1 antibody upon initiation of IgA immune complex-induced alveolitis resulted in a marked reduction in lung injury as quantified by pulmonary vascular permeability and pulmonary hemorrhage indices (Fig. 7; Table I). Analysis of BAL contents 4 h after anti-MCP 1 infusion revealed a nearly 80% reduction in retrievable mononuclear phagocytes compared with control animals (Table II). These data suggest that MCP 1 is required for the full development of IgA immune complex-induced alveolitis. The morphometric and cell retrieval data indicate that MCP 1 is required for maximum pulmonary monocyte/macrophage recruitment.

DISCUSSION

Based on in vitro studies that indicate that MCP 1/JE triggers monocyte chemotaxis and activation, and more recently, studies that have revealed either increased MCP 1/JE mRNA levels or immunoreactive MCP 1 monocyte chemotactic activity in atherosclerotic lesions (11, 12), granulomas

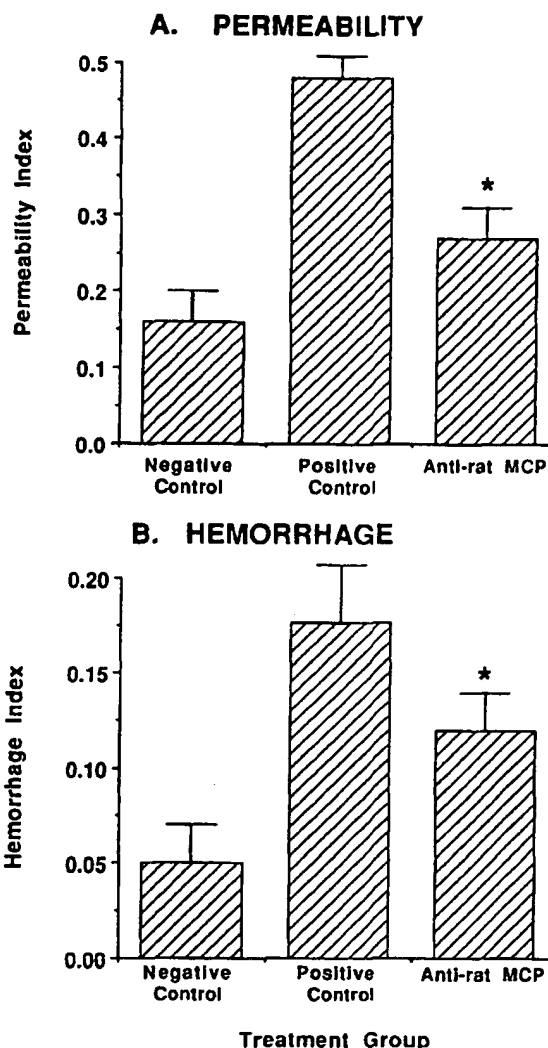


Figure 7. Blockade of IgA immune complex lung injury with anti-MCP 1 antibodies. Anti-MCP 1 (0.5 ml) was infused i.v. upon initiation of lung injury. Positive control rats were treated identically but received equivalent quantities of preimmune rabbit serum (0.5 ml) in place of anti-MCP 1. Negative control rats received intratracheal IgA anti-DNP/BSA but no i.v. Ag. Lung injury was quantitated by determining permeability indices (A) and hemorrhage indices (B) 4 h after instillation of anti-DNP/BSA as previously described (1-4). These data represent means \pm SEM of two experiments in which five rats per variable were employed. The data were analyzed by one-way analysis of variance with significance assigned for $p < 0.05$ (37). *Indicates a significant difference vs positive controls.

TABLE I
Morphometric analysis of IgA immune complex alveolitis: effect of anti-MCP 1 antibodies^a

Intervention	Mononuclear Phagocyte Influx		Alveolar Hemorrhage	
	M Φ /40X HPF	p ^b	RBC/40X HPF	p ^b
A. Preimmune serum (0.5 ml) (positive control)	37 \pm 5		23 \pm 4	
B. Anti-MCP 1 serum (0.5 ml)	18 \pm 3	<0.05 vs A	11 \pm 3	<0.05 vs A
C. None (negative control)	13 \pm 3		4 \pm 1	

^a Immune complex alveolitis and anti-MCP 1 interventions were carried out as described in Figure 7.

^b One-way analysis of variance (37).

(13), and transplanted murine melanomas infiltrated with macrophages (30), it has been suggested that MCP 1/JE mediates monocyte/macrophage-rich pathologic processes. The present study suggests that MCP 1/JE

TABLE II
Effect of anti-MCP 1 on alveolar macrophage retrieval in IgA
alveolitis^a

Treatment	Macrophages Retrieved	% of Total Cells	% Reduction ^b
A. None (negative control)	$4.2 \pm 0.62 \times 10^6$	93%	
B. Preimmune serum (0.5 ml; i.v.)	$9.6 \pm 0.83 \times 10^6$	89%	
C. Anti-MCP 1 serum (0.5 ml; i.v.)	$5.3 \pm 0.47 \times 10^6$	92%	79.7% vs B

^a Immune complex alveolitis and anti-MCP 1 interventions were carried out as described in Figure 7.

^b One-way analysis of variance (37).

may play an important role in the development of monocyte/macrophage-dependent IgA immune complex alveolitis in the rat. Infusion of antibodies directed against rat MCP 1 into rats with developing IgA immune complex-induced alveolitis resulted in a marked decrease in lung injury.

Critical to this study was to clone and express rat MCP 1. We chose the baculovirus/insect cell expression system because it has been successfully used to express other eukaryotic genes as nonfusion proteins (reviewed in Reference 29). Proteins produced in this expression system undergo post-translational processing and have been obtained in relatively large amounts (reviewed in Reference 29). We cloned rat MCP 1 cDNA from rat pulmonary artery endothelium stimulated with TNF- α because others have observed that TNF- α -stimulated human endothelial cells produce MCP 1 (26–28) and because we had previously detected markedly increased levels of rat MCP 1 mRNA in TNF- α -stimulated rat endothelium (M. L. Jones and J. S. Warren, unpublished data). Sf-21 cells infected with AcMCP 1 produced relatively large quantities of biologically inactive rat MCP 1. Solubilization and refolding of MCP 1 contained in Sf-21 cell lysate pellets yielded monocyte-specific chemotactic activity.

Yoshimura et al. recently cloned rat MCP 1 cDNA from Con A-stimulated rat spleen cells (18). The rat MCP 1 cDNA sequence published by Yoshimura et al. is in agreement with the sequence of rat JE genomic DNA reported by Timmers et al. (31). The deduced amino acid sequence of rat MCP 1 bears a strong degree of homology to both human and mouse MCP 1 (32–34). In the present study, SDS/PAGE analysis indicated that the rat MCP 1 species expressed in the baculovirus/insect cell expression system have m.w. of approximately 18 kDa, 21 kDa, and 23 kDa. A shift in the predominant protein species from 23 kDa (and to a lesser extent, 21 kDa), to 16- to 18-kDa species in tunicamycin-treated Sf-21 cell cultures suggests that the two larger species are glycosylated. A m.w. of 16 to 18 kDa agrees with the m.w. of unprocessed rat MCP 1 predicted by its open reading frame (18). Yoshimura et al. reported a 14-kDa monocyte-specific chemotactic activity in the supernatants of rat malignant fibrous histiocytoma cell lines (MFR 11) that express high levels of MCP 1 mRNA (18). A m.w. of 14 kDa approximates the predicted size of mature secreted (cleaved) rat MCP 1 in which the N terminus appears to be the glutamine located at position 24 (18). Amino acid sequence data from mature native human MCP 1 indicates that it starts with a glutamine at position 24. The predicted N-terminal amino acid sequence of unprocessed rat MCP 1 is hydrophobic, typical of a signal peptide, and consistent

with the observation that MCP 1 is a secreted protein.

As reported for various other proteins expressed in baculovirus insect cells (35), rat rMCP 1 was biologically inactive until it was denatured (in 8 M urea and 10 mM 2-ME) and then refolded by dialysis against cold HBSS containing 1 mM 2-ME. The refolded 21-kDa and 23-kDa rat MCP 1 species produced in this study were active in the monocyte chemotaxis assay at concentrations of approximately 35 ng/ml. Although the proportion of rat MCP 1 that is active could not be determined precisely, comparison with the reported potency of native human MCP 1 (optimal activity 10^{-9} M) suggests that only a small proportion of rat MCP 1 (<1%) is active (9, 10). It should be emphasized that this is only an approximation because native human MCP 1 and rat MCP 1 expressed using the baculovirus-insect cell system were not directly compared and because interspecies differences may influence activity quantitated by the monocyte chemotaxis assay. It is presently unknown exactly how polypeptides are produced or stored in a soluble form *in vivo* but several mechanisms may be operative. It is possible that insoluble aggregate formation is favored in insect cells that are overexpressing a foreign protein such as MCP 1. Aggregate formation may be caused by strong hydrophobic and/or ionic interactions among nascent peptides. Alternatively, normal protein folding may require so-called chaperones, proteins that regulate correct self-assembly of nascent peptides without themselves becoming incorporated (reviewed in Reference 36). Little is known about the secretion of native MCP 1 except that it appears to be processed via an N-terminal hydrophobic signal sequence (31–34). Finally, we cannot conclude from this study whether the monocyte chemotactic activity observed in the 21- and 23-kDa species (in contrast to the 18-kDa species) is a function of glycosylation or more efficient refolding.

Provision of MCP 1/JE-specific antibody was paramount to this study. As noted in Figure 5, rabbit polyclonal antibody raised against the 23-kDa rat rMCP 1 species reacted with the 18-kDa, 21-kDa, and 23-kDa baculovirus expression products as would be predicted if these represent variably glycosylated rat MCP 1 species. Anti-rat MCP 1 blocked MCP 1-triggered monocyte chemotaxis in a dose-dependent manner (Fig. 6). Finally, anti-MCP 1 selectively blocked monocyte-specific chemotactic activity secreted by TNF- α -stimulated rat endothelial cells. This observation, supported by the facts that TNF- α -stimulated rat endothelial cells, like human endothelial cells, express increased levels of MCP 1 mRNA (data not shown), that the monocyte-specific chemotactic activity is present in serum-free medium, and that the monocyte chemotactic activity has a m.w. between 8 and 30 kDa, provides compelling evidence that anti-MCP 1 recognizes and neutralizes native rat MCP 1.

The *in vivo* data indicate that MCP 1 is required for full development of IgA immune complex-induced pulmonary vascular leakage and hemorrhage. The lung lavage data (Table II) suggest that MCP 1 may play a role in recruitment of mononuclear phagocytes into the alveolar space. However, it is unclear whether this is a direct effect of local MCP 1 elaboration or a sequel to acute tissue injury *per se*. Attempts to measure MCP 1 activity in BAL fluid and serum were unsuccessful suggesting either that the amounts produced were below the level of detection by

bioassay (10^{-10} M) or that MCP 1 is catabolized, complexed, or otherwise sequestered. It is also possible that MCP 1 is produced chiefly by the endothelium and thus cannot be measured by the assay methods in hand. The relative importance of MCP 1 as a chemotactic factor and as a monocyte/macrophage-activating factor remain to be determined in this model. Despite the issues to be addressed, these data indicate that MCP 1/JE plays an obligate role in the pathogenesis of IgA immune complex alveolitis in the rat.

Acknowledgments. The authors thank Dr. Chris Richardson and Dr. Jorge Vialard for providing the pJVETLZ transfer vector and for discussions regarding the baculovirus expression system, Dr. Teizo Yoshimura for the rat MCP 1 cDNA sequence before it was published, Cynthia Shanteau for manuscript preparation, and Robin Kunkel for figure preparation.

REFERENCES

- Johnson, K. J., B. S. Wilson, G. O. Till, and P. A. Ward. 1984. Acute lung injury in rat caused by immunoglobulin A immune complexes. *J. Clin. Invest.* 74:358.
- Johnson, K. J., P. A. Ward, R. G. Kunkel, and B. S. Wilson. 1986. Mediation of IgA induced lung injury in the rat: role of macrophages and reactive oxygen products. *Lab. Invest.* 54:499.
- Warren, J. S., R. G. Kunkel, R. H. Simon, K. J. Johnson, and P. A. Ward. 1989. Ultrastructural cytochemical analysis of oxygen radical-mediated immunoglobulin A immune complex induced lung injury in the rat. *Lab. Invest.* 60:651.
- Warren, J. S., P. A. Barton, and M. L. Jones. 1991. Contrasting roles for tumor necrosis factor in the pathogenesis of IgA and IgG immune complex lung injury. *Am. J. Pathol.* 138:581.
- Rollins, B. J., P. Stier, T. E. Ernst, and G. G. Wong. 1989. The human homologue of the JE gene encodes a monocyte secretory protein. *Mol. Cell. Biol.* 9:4687.
- Cochran, B. H., A. C. Reffel, and C. D. Stiles. 1983. Molecular cloning of gene sequence regulated by platelet-derived growth factor. *Cell* 33:939.
- Leonard, E. J., and T. Yoshimura. 1990. Human monocyte chemoattractant protein 1 (MCP 1). *Immunol. Today* 11:97.
- Matsushima, K., C. G. Larsen, G. C. DuBois, and J. J. Oppenheim. 1989. Purification and characterization of a novel monocyte chemotactic and activating factor produced by a human myelomonocytic cell line. *J. Exp. Med.* 169:1485.
- Yoshimura, T., E. A. Robinson, S. Tanaka, E. Appella, J. Kuratsu, and E. J. Leonard. 1989. Purification and amino acid analysis of two human glioma-derived monocyte chemoattractants. *J. Exp. Med.* 169:1449.
- Yoshimura, T., E. A. Robinson, S. Tanaka, E. Appella, and E. J. Leonard. 1989. Purification and amino acid analysis of two human monocyte chemoattractants produced by phytohemagglutinin-stimulated human blood mononuclear leukocytes. *J. Immunol.* 142:1956.
- Yla-Herttuala, S., B. A. Lipton, M. E. Rosenfield, T. Sarkioja, T. Yoshimura, E. J. Leonard, J. L. Witztum, and D. Steinberg. 1991. Expression of monocyte chemoattractant protein 1 in macrophage-rich areas of human and rabbit atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA* 88:5252.
- Nelkenna N. A., S. R. Coughlin, D. Gordon, and J. L. Wilcox. 1991. Monocyte chemoattractant protein-1 in human atheromatous plaques. *J. Clin. Invest.* 88:1121.
- Jones, M. L., and J. S. Warren. 1991. Monocyte chemoattractant protein 1 in a rat model of pulmonary granulomatosis. *Lab. Invest.* 66:498.
- Vialard, J., M. Lalviere, T. Vernet, D. Briedis, G. Alkhatib, D. Henning, D. Levin, and C. Richardson. 1990. Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novel baculovirus vector containing the beta-galactosidase gene. *J. Virol.* 64:37.
- Ginsburg, I., D. F. Gibbs, L. Schuger, K. J. Johnson, V. S. Ryan, P. A. Ward, and J. Varani. 1989. Vascular endothelial cell killing by combinations of membrane-active agents and hydrogen peroxide. *Free Rad. Biol. Med.* 7:369.
- Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Lab., Cold Spring Harbor, NY, pp. 73-82.
- Gubler, U., and B. J. Hoffman. 1983. A simple and very efficient method for generating cDNA libraries. *Gene* 25:263.
- Yoshimura, T., M. Takeya, and K. Takahashi. 1991. Molecular cloning of rat monocyte chemoattractant protein 1 (MCP 1) and its expression in rat spleen cells and tumor cell lines. *Biochem. Biophys. Res. Commun.* 174:504.
- Summers, M. D., and O. E. Smith. 1988. A manual of methods for baculovirus vectors and insect cell culture procedures. *Texas Agric. Exp. Stn. Bull. No. 1555*.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature* 227:680.
- Wray, W., T. Boulikas, V. Wray, and R. Hancock. 1981. Silver staining of proteins in polyacrylamide gels. *Analyt. Biochem.* 118:197.
- Falk, W., R. H. Goodwin, Jr., and E. J. Leonard. 1980. A 48-well micro chemotaxis assembly for rapid and accurate measurement of leukocyte migration. *J. Immunol. Methods* 33:329.
- Visser, M. C. M., S. A. Jester, and J. C. Fantone. Rapid purification of human peripheral blood monocytes by centrifugation through Ficoll-Hypaque and Sepacell-MN. *J. Immunol. Methods* 110:203.
- Boyum, A. 1968. Separation of leukocytes from blood and bone marrow. *Scand. J. Clin. Lab. Invest.* 21:77.
- Eisen, H. N. 1964. Preparation of purified anti-2,4-dinitrophenol antibodies. *Methods Med. Res.* 10:94.
- Strieter, R. M., R. Wiggins, S. Phan, B. L. Wharram, H. J. Showell, D. G. Remick, S. W. Chensue, and S. L. Kunkel. 1989. Monocyte chemotactic protein gene expression by cytokine-treated human fibroblasts and endothelial cells. *Biochem. Biophys. Res. Commun.* 162:694.
- Dixit, V. M., S. Green, V. Sarma, L. B. Holzman, F. W. Wolf, K. O. O'Rourke, P. A. Ward, E. V. Prochownik, and R. M. Marks. 1990. Tumor necrosis factor- α induction of novel gene products in human endothelial cells including a macrophage-specific chemotaxin. *J. Biol. Chem.* 265:2973.
- Rollins, B. J., T. Yoshimura, E. J. Leonard, and J. S. Pober. 1990. Cytokine-activated human endothelial cells synthesize and secrete a monocyte chemoattractant, MCP 1/JE. *Am. J. Pathol.* 136:1229.
- Luckow, V. A., and M. D. Summers. 1988. Trends in the development of baculovirus expression vectors. *Bio/Technology* 6:47.
- Botlazzi, B., S. Walter, D. Govani, F. Colotta, and A. Mantovani. 1992. Monocyte chemotactic cytokine gene transfer modulates macrophage infiltration, growth, and susceptibility to IL-2 therapy of a murine melanoma. *J. Immunol.* 148:1280.
- Timmers, H. T. M., G. J. Pronk, J. L. Box, and A. J. van der Eb. 1990. Analysis of the rat JE gene promoter identities on AP-1 binding site essential for basal expression but not for TPA induction. *Nucleic Acids Res.* 18:23.
- Robinson, E. A., T. Yoshimura, E. J. Leonard, S. Tanaka, P. R. Griffin, J. Shabanowitz, D. F. Hunt, and E. Appella. 1989. Complete amino acid sequence of a human monocyte chemoattractant, a putative mediator of cellular immune reactions. *Proc. Natl. Acad. Sci. USA* 86:1850.
- Yoshimura, T., N. Yuhki, S. K. Moore, E. Appella, M. I. Lerman, and E. J. Leonard. 1989. Human monocyte chemoattractant protein-1 (MCP-1): full length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. *FEBS Lett.* 244:487.
- Rollins, B. J., E. D. Morrison, and C. D. Stiles. 1988. Cloning and expression of JE, a gene inducible by platelet-derived growth factor and whose product has cytokine-like properties. *Proc. Natl. Acad. Sci. USA* 85:3738.
- Berndt, N., and P. T. W. Cohen. 1990. Renaturation of protein phosphatase 1 expressed at high levels in insect cells using a baculovirus vector. *Eur. J. Biochem.* 190:291.
- Ellis, R. J., and S. M. Hemmingsen. 1989. Molecular chaperones: proteins essential for the biogenesis of some macromolecular structures. *Trends Biochem. Sci.* 14:339.
- Linton, M., and P. S. Gallo, Jr. 1975. *The Practical Statistician: Simplified Handbook of Statistics*. Brooks-Cole Publishing Co., Monterey, CA, pp. 136-218.